

Substrate Stress Relaxation Regulates Cell Migration

by

Yi Zuo

**A thesis submitted to The Johns Hopkins University
in conformity with the requirements for the degree of
Master of Science in Engineering**

Baltimore, Maryland

May, 2019

© 2019 by Yi Zuo

All rights reserved

Abstract

Mechanical stimuli from the extracellular matrix (ECM) play an essential role in regulating important biological processes including cell spreading, migration, proliferation, and differentiation. However, these studies typically use elastic materials as substrates, whereas natural ECM is a viscoelastic material and exhibits stress relaxation properties. Here, we investigate the influence of substrate stress relaxation on cell migration properties by using an alginate-based materials system with adjustable stress relaxation rates. We find that two-dimensional (2D) collective cell migration, three-dimensional (3D) single cell migration and three-dimensional (3D) collective cell migration are all enhanced when cells were cultured in gels with fast relaxation. However, even though the two-dimensional (2D) single cell average migration speed initially increases with the increase of stress relaxation rates, it later decreases. These findings highlight stress relaxation as a key factor regulating cell migration.

Primary Reader: Luo Gu

Thesis Committee

Primary Readers

Luo Gu (Primary Advisor)
Assistant Professor
Department of Material Science and Engineering
Johns Hopkins Whiting School of Engineering

Acknowledgments

First, I would like to express my deep gratitude to my advisor Prof. Gu for giving me the opportunity to join the lab and providing continuous support of my Master's study and related research. His sincerity, motivation and knowledge have inspired me.

Besides my advisors, my sincere thanks also go to my fellow labmates: Joo Ho Kim, Joon Eoh, Supeng Ding, Zhiwei Fang, Yahong Zhao, Sabrina Shen and Diane Lee for their help, cooperation and encouragement.

Last but not the least, I would like to thank my parents and my boyfriend for supporting me spiritually throughout writing this thesis and my life in general.

Table of Contents

Table of Contents	v
List of Tables	vii
List of Figures	ix
1 Introduction	1
1.1 Cell Migration	1
1.2 Alginate Hydrogels	3
1.3 Mechanotransduction	5
2 Methods	11
2.1 Alginate Hydrogels System	11
2.1.1 Alginate Preparation	11
2.1.2 Alginate Characterization	12
2.1.3 Alginate Hydrogel Fabrication	12
2.1.4 Mechanical Characterization	12
2.2 2D Single Cell Tracking	13

2.3	2D Wound Model	14
2.4	3D Single Cell Tracking	15
2.5	3D Radial Cell Migration	16
3	Results	18
3.1	Alginate Hydrogels System	18
3.2	2D Single Cell Tracking	20
3.3	2D Wound Model	24
3.4	3D Single Cell Tracking	27
3.5	3D Radial Cell Migration	29
4	Discussion	37
4.1	Alginate Hydrogels System	39
4.2	2D Single Cell Tracking	41
4.3	2D Wound Model	43
4.4	3D Single Cell Tracking	45
4.5	3D Radial Cell Migration	47
5	Conclusion and Future Work	57

List of Tables

3.1	Molecular Weight for four types of alginates used in experiments. Based on the results from GPC, the MW range of the alginates is from 260kPa to 27kPa.	20
3.2	Designed Ca^{2+} concentration in final gels and half time ($\tau_{1/2}$) for different hydrogels. Combined alginates with appropriate MW and Ca^{2+} concentration were able to form alginate hydrogels with different stress relaxation properties but similar elastic modulus.	24
3.3	Quantification of average speed for 2D single cell migration with different mechanical properties. The average speed of 2D single cell migration increased first then decreased with the increase of stress relaxation rates.	26
3.4	2D Wound Model. The percentage of wound closure after 19h increased with the decreasing of elastic modulus of alginate hydrogels and also increased with the increase of stress relaxation rate.	26

3.5	Average speed for 3D single cell migration. 3D single cell migration rate was enhanced by increasing the stress relaxation rate.	27
3.6	Average length of longest protrusions for 3 different stiffness hydrogels conditions with fast stress relaxation rates. Higher the elastic modulus of alginate hydrogels, shorter the average length of the longest protrusions.	34

List of Figures

2.1	Examples processed images by Imagej software at 0 h and 19 h for 2D wound model. The edge of the wound area was precisely defined by the Imagej software.	15
3.1	Elastic modulus measurements of gels targeting for 7kPa. Elastic modulus differences between four groups are not significant.	19
3.2	Elastic modulus measurements of gels targeting for 12kPa. Elastic modulus differences between four groups are not significant.	19
3.3	Elastic modulus measurements of gels targeting for 17kPa. Elastic modulus differences between four groups are not significant.	21
3.4	The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 7kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.	21

3.5	The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 12kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.	22
3.6	The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 17kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.	23
3.7	Quantification of average speed for 2D single cell migration with different mechanical properties. The average speed of 2D single cell migration increased first then decreased with the increase of stress relaxation rate or decreasing of half time $\tau_{1/2}$. *indicates $p < 0.05$ (Student's t-test), **indicates $p < 0.01$ (Student's t-test).	25
3.8	Examples images at 0 h and 19 h for 2D wound model. (a) A regular and consistent gap was created on soft hydrogel surface without altering the soft hydrogel substrate by the method we developed. (b) After 19 hours, part of the gap was closed by cells.	27
3.9	Quantification of average speed for 3D single cell migration with different stress relaxation rate. When holding the elastic modulus constant and final cell concentration, the average speed of 3D single cell migration was suppressed with the increase of stress relaxation rate or decreasing of half time $\tau_{1/2}$.	28

3.10	Example images of two 3D radial cell migration patterns. (a) Radially symmetric migration of individual cells;(b) Formation of protrusions (Khain2006).	29
3.11	Examples images of cell spheroids. (a) Regular and designed size of cell spheroids were made by Aggrewell™ 800 24-well Plate. (b) Confocal images of one cell spheroid.	30
3.12	Example confocal image of 3D radial cell migration. NIH/3T3-GFP cell spheroids showed 3D radial protrusion pattern when encapsulated in alginate hydrogels.	30
3.13	Examples images showed the growth of spheroids protrusions with time. There were obvious 3D radial protrusion migration phenomenon when cell spheroids were encapsulated in I1G-8 Mrad hydrogels with 7 kPa stiffness, with fast relaxation rate and low stiffness.	31
3.14	Representative images of NIH/3T3-GFP spheroids encapsulated within alginate gels with the indicated relaxation time $\tau_{1/2}$ and two elastic modulus (7 kPa and 17 kPa). Images were taken after three days in culture. Scale bar is 300 μm . There was only obvious protrusion migration in fast stress relaxation conditions (I1G-8 Mrad).	32

3.15	Examples images of 3D radial migration phenomenon for 3 different stiffness hydrogels conditions with fast stress relaxation rates. Images were taken after 9 days in culture.(a)Alginate hydrogels with 7 kPa elastic modulus had the high length and density of protrusions. (b)Alginate hydrogels with 12 kPa elastic modulus had the medium length and density of protrusions. (c)Alginate hydrogels with 17 kPa elastic modulus had the low length and density of protrusions.	33
3.16	Quantification of the average length of longest protrusions for 3 different stiffness hydrogels conditions with fast stress relaxation rates. Stiffer the alginate hydrogels, shorter the average length of the longest protrusions. *indicates $p < 0.05$ (Student's t-test), **indicates $p < 0.01$ (Student's t-test), ***indicates $p < 0.001$ (Student's t-test).	33

Chapter 1

Introduction

1.1 Cell Migration

Cell migration is defined as any directed movement of a single cell, cell monolayer or cell cluster from one location to another (Grada et al., 2017). It is an essential component for many processes involved in the maintenance and development of multi-cellular organisms including embryonic development, wound healing, cancer invasion and immune responses (Grada et al., 2017; Trepac, Chen, and Jacobson, 2012; Fallis, 2013; Even-Ram and Yamada, 2005). The capability to migrate allows cells to alter their position within tissues or between different organs in order to respond to various stimuli.

The basic biological processes involved in cell migration throughout the extracellular matrix (ECM) can be summarized into several stages (Lauffenburger and Horwitz, 1996; Decaestecker et al., 2006). Localized actin polymerization forms filaments and the asymmetric distribution of signaling molecules including PI3Ks, PTEN, and Rho GTPases (Sugawara et al., 2016), which initial

cell polarization. This lead to the extension of cytoplasmic protrusions or pseudopods. Focal adhesions then form through interactions between the cell adhesion receptors on the surface of the protrusions and various ligands within the ECM. Matrix-degrading proteases, such as the matrix metalloproteinases (MMP) causes degradation of the matrix and allow for forward-expansion of the cell body. An actin filaments then interact with the cross-linking and contractile proteins, such as myosin, which stabilize and contract the actin strands. These contractions produce the forward movement and translocation along the substrate.

The two most commonly understood patterns of cell migration include single cell and collective cell migration (Decaestecker et al., 2006; Grada et al., 2017). Collective cell migration is defined as the coordinated movement among a group of cells that still keep the inter-cellular connections and collective polarity between cells (Grada et al., 2017). Single cell migration describes migration with no cell-cell contact (Decaestecker et al., 2006).

Furthermore, depending on cytoskeletal structure, cell types and the context in which it is migrating, single cell migration mechanisms can be classified into mesenchymal motility modes and amoeboid motility modes (Decaestecker et al., 2006; Grada et al., 2017). In general, cells in mesenchymal modes follow the five-stage migration previously mentioned, while amoeboid-like cells have relatively weak interactions with the matrix and use a fast "crawling" type of movement (Decaestecker et al., 2006).

Many studies have showed that mechanical stimuli, like stiffness, stress relaxation and topography are essential factors to control the migration of

cells (Pelham, And, and Wang, 1998; Sugawara et al., 2016; Lou et al., 2018). Interestingly, some studies have showed the increased hydrostatic pressure between nucleus and cell edge can cause lamellipodia-independent 3D cell migration (Petrie, Koo, and Yamada, 2014).

However, there are not comprehensive studies on the effects of stress relaxation on cell migration using different cell migration models. Therefore, we established a hydrogel system to investigate the influence of stress relaxation on cell migration.

1.2 Alginate Hydrogels

Alginate is one type of polysaccharides derived from brown seaweed containing linear 1,4 linked residues of β -D-mannuronic acid (M) and C5-epimer α -L-guluronic acid (G) (Augst, Kong, and Mooney, 2006). The molecular structure is a copolymer consisting of alternating blocks of consecutive G or M monomers (-GGG- or -MMM-) or blocks of alternating monomers (-MGMG-) (Augst, Kong, and Mooney, 2006; Lee and Mooney, 2012). The blocks vary considerably in distribution and length, depending on the species and the part of the seaweed used for alginate extraction. The diversity in chemical composition and distribution of blocks in alginate allows for the production of gels with a wide variety of physical properties (Augst, Kong, and Mooney, 2006; Lee and Mooney, 2012).

Alginate can be ionically cross-linked with divalent cations to form hydrogels. It has been shown to have high affinity for alkaline earth metals

(except Mg^{2+}) (Donati et al., 2005; Seely and Hart, 1974; Haug and Smidsrod, 1970). Due to a cooperative binding of consecutive residues in different alginate chains, chelation of the gel-forming ion happens between two consecutive residues and an intermolecular gel network is formed (Andersen, Auk-Emblem, and Dornish, 2015). The selection of type and amount of gel forming ions will influence the resulting properties of the hydrogels. When increasing G-content, length of G-blocks and molecular weight (MW), the gel elasticity, stability and porosity also increase (Lee and Mooney, 2012; Gombotz and Wee, 1998; Ivan Donati and Skjak-Brak, 2005).

Alginate hydrogels have been widely used as biomaterials, such as scaffolds for tissue engineering, delivery vehicles for drugs, and extracellular matrices (ECM) for basic biological studies (Ahearne, Yang, and Liu, 2008; Lee and Mooney, 2012; Augst, Kong, and Mooney, 2006; Andersen, Auk-Emblem, and Dornish, 2015). The material properties of these alginate hydrogels such as mechanical stiffness, swelling, cell attachment, biodegradability and binding affinity were controlled by chemical or physical modifications to achieve these applications (Rowley, Madlambayan, and Mooney, 1999).

Alginate hydrogels have a number of advantages over synthetic ones, such as biocompatibility, ease of fabrication, viscoelastic properties and non-toxicity (Lee and Mooney, 2012; Ahearne, Yang, and Liu, 2008). The mechanical properties of alginate hydrogels are highly dependent on the characteristics of the polymer, the gelling environment, and the crosslinking method (Ahearne, Yang, and Liu, 2008; Lee and Mooney, 2012). Specifically, the stress-strain behavior of alginate hydrogels with ionic crosslinks is time-dependent, due

to the intrinsic viscoelastic properties of the ionically cross-linked hydrogels (Zhao et al., 2010).

Based on Recent works, it is able to control stress relaxation rates of alginate hydrogels, independent of stiffness, ligand density and polymer concentration (Chaudhuri et al., 2016), which makes it possible for us to study the influences of stress relaxation to cell migration.

1.3 Mechanotransduction

Mechanotransduction or mechanobiology represents the ability of cells to sense and respond to mechanical stimuli by converting these stimuli to biochemical signals (Paluch et al., 2015). It is a rapidly progressing field, and is essential for a broad range of biological and physiological process including proliferation, differentiation and cell migration (Paluch et al., 2015; Dupont et al., 2011).

Comprehensive research over the past decades have showed that several molecular players, including calcium and other ions, and cell-signalling molecules and transcription factors, that involved in cellular mechanotransduction (Jalouk and Lammerding, 2009). Based on previous studies, the mechanical properties of the substrates play a critical role in cellular behavior and mechanotransduction. These force-induced responses initiate a wide variety of signaling pathways related to gene expression, protein synthesis and changes to cellular phenotype (Goldmann, 2014). There have been studies showing the effects of matrix stiffness to cytoskeleton, cell-matrix adhesions,

cell division, migration and apoptosis (Goldmann, 2014). Studies in mechanotransduction often focus on sensory cells, cells involved in the sense reception of touch or hearing, that have specific structures can alter mechanical inputs into biochemical signals (Jaalouk and Lammerding, 2009). Recently, there are also studies about the mechanotransduction on different cell types such as endothelial cells, myocytes and vascular smooth muscle cells (Engler et al., 2006). Also, recent studies used RNA-seq mapping to explore the changes of transcriptional programs of mesenchymal stem cells (MSCs) encapsulated in alginate hydrogels, which allowed for independent control of matrix stiffness, stress relaxation and ligand density (Darnell, Gu, and Mooney, 2018; Darnell et al., 2018). They found a great number of genes were regulated by physical microenvironment, like stiffness (Darnell, Gu, and Mooney, 2018; Darnell et al., 2018).

Here we investigate the influence of stress relaxation on single cell migration and collective cell migration in both 2D culture and 3D culture.

References

- Grada, Ayman, Marta Otero-Vinas, Francisco Prieto-Castrillo, Zaidal Obagi, and Vincent Falanga (2017). "Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay". In: *Journal of Investigative Dermatology* 137.2, e11–e16. ISSN: 15231747. DOI: [10.1016/j.jid.2016.11.020](https://doi.org/10.1016/j.jid.2016.11.020). URL: <http://dx.doi.org/10.1016/j.jid.2016.11.020>.
- Trepap, Xavier, Zaozao Chen, and Ken Jacobson (2012). "Cell migration." In: *Comprehensive Physiology* 2.4, pp. 2369–92. ISSN: 2040-4603. DOI: [10.1002/cphy.c110012](https://doi.org/10.1002/cphy.c110012). URL: <http://www.ncbi.nlm.nih.gov/pubmed/23720251><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4457291>.
- Fallis, a.G (2013). *Cell migration*. Vol. 53. 9, pp. 1689–1699. ISBN: 9788578110796. DOI: [10.1017/CB09781107415324.004](https://doi.org/10.1017/CB09781107415324.004). arXiv: [arXiv:1011.1669v3](https://arxiv.org/abs/1011.1669v3).
- Even-Ram, Sharona and Kenneth M. Yamada (2005). "Cell migration in 3D matrix". In: *Current Opinion in Cell Biology* 17.5, pp. 524–532. ISSN: 09550674. DOI: [10.1016/j.ceb.2005.08.015](https://doi.org/10.1016/j.ceb.2005.08.015). URL: <http://linkinghub.elsevier.com/retrieve/pii/S0955067405001183>.
- Lauffenburger, Douglas A. and Alan F. Horwitz (1996). "Cell migration: A physically integrated molecular process". In: *Cell* 84.3, pp. 359–369. ISSN: 00928674. DOI: [10.1016/S0092-8674\(00\)81280-5](https://doi.org/10.1016/S0092-8674(00)81280-5).
- Decaestecker, Christine, Olivier Debeir, Philippe Van Ham, and Robert Kiss (2006). "Can Anti-Migratory Drugs Be Screened InVitro ? A Review of 2D and 3D Assays for the Quantitative Analysis of Cell Migration". In: 27.2, pp. 149–176. DOI: [10.1002/med.20078](https://doi.org/10.1002/med.20078).
- Sugawara, Michiko, Hiromi Miyoshi, Takuya Miura, Hiroto Tanaka, Ken Ichi Tsubota, and Hao Liu (2016). "Dynamics of Actin Stress Fibers and Focal Adhesions during Slow Migration in Swiss 3T3 Fibroblasts: Intracellular Mechanism of Cell Turning". In: *BioMed Research International*. ISSN: 23146141. DOI: [10.1155/2016/5749749](https://doi.org/10.1155/2016/5749749).

- Pelham, Robert J, J R And, and Yu-Li Wang (1998). *Cell Locomotion and Focal Adhesions Are Regulated by the Mechanical Properties of the Substrate*. Tech. rep., pp. 348–350. URL: <http://www.journals.uchicago.edu/t-and-c>.
- Lou, Junzhe, Ryan Stowers, Sungmin Nam, Yan Xia, and Ovijit Chaudhuri (2018). “Stress relaxing hyaluronic acid-collagen hydrogels promote cell spreading, fiber remodeling, and focal adhesion formation in 3D cell culture”. In: *Biomaterials* 154, pp. 213–222. ISSN: 18785905. DOI: [10.1016/j.biomaterials.2017.11.004](https://doi.org/10.1016/j.biomaterials.2017.11.004). URL: <https://doi.org/10.1016/j.biomaterials.2017.11.004>.
- Petrie, Ryan J, Hyun Koo, and Kenneth M Yamada (2014). “Generation of compartmentalized pressure by a nuclear piston governs cell motility in a 3D matrix.” In: *Science (New York, N.Y.)* 345.6200, pp. 1062–5. ISSN: 1095-9203. DOI: [10.1126/science.1256965](https://doi.org/10.1126/science.1256965). URL: <http://www.ncbi.nlm.nih.gov/pubmed/25170155><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5248932>.
- Augst, Alexander D., Hyun Joon Kong, and David J. Mooney (2006). “Alginate hydrogels as biomaterials”. In: *Macromolecular Bioscience* 6.8, pp. 623–633. ISSN: 16165187. DOI: [10.1002/mabi.200600069](https://doi.org/10.1002/mabi.200600069).
- Lee, Kuen Yong and David J. Mooney (2012). “Alginate: Properties and biomedical applications”. In: *Progress in Polymer Science* 37.1, pp. 106–126. ISSN: 0079-6700. DOI: [10.1016/J.PROGPOLYMSCI.2011.06.003](https://doi.org/10.1016/J.PROGPOLYMSCI.2011.06.003). URL: <https://www.sciencedirect.com/science/article/pii/S0079670011000918>.
- Donati, Ivan, Synnøve Holtan, Yrr A Mørch, Massimiliano Borgogna, Mariella Dentini, and Gudmund Skjak-Braek (2005). “New hypothesis on the role of alternating sequences in calcium-alginate gels.” In: *Biomacromolecules* 6.2, pp. 1031–40. ISSN: 1525-7797. DOI: [10.1021/bm049306e](https://doi.org/10.1021/bm049306e). URL: <https://pubs.acs.org/doi/10.1021/bm049306e><http://www.ncbi.nlm.nih.gov/pubmed/15762675>.
- Seely, G R and R L Hart (1974). “The binding of alkaline earth metal ions to alginate.” In: *Macromolecules* 7.5, pp. 706–10. URL: <http://www.ncbi.nlm.nih.gov/pubmed/4418138>.
- Haug, Arne and Olav Smidsrod (1970). “Selectivity of some anionic polymers for divalent metal ions”. In: *Acta chem. scand* 24.3, pp. 843–854.
- Andersen, Therese, Pia Auk-Emblem, and Michael Dornish (2015). “3D Cell Culture in Alginate Hydrogels”. In: *Microarrays* 4.2, pp. 133–161. ISSN: 2076-3905. DOI: [10.3390/microarrays4020133](https://doi.org/10.3390/microarrays4020133). URL: <http://www.mdpi.com/2076-3905/4/2/133/>.

- Gombotz, Wayne R and SiowFong Wee (1998). "Protein release from alginate matrices". In: *Advanced Drug Delivery Reviews* 31.3, pp. 267–285. ISSN: 0169-409X. DOI: [10.1016/S0169-409X\(97\)00124-5](https://doi.org/10.1016/S0169-409X(97)00124-5). URL: <https://www.sciencedirect.com/science/article/pii/S0169409X97001245>.
- Ivan Donati Synnove Holtan, Yrr A. Morch Massimiliano Borgogna Mariella Dentini and Gudmund Skjak-Brak (2005). "New Hypothesis on the Role of Alternating Sequences in Calcium Alginate Gels". In: DOI: [10.1021/BM049306E](https://doi.org/10.1021/BM049306E). URL: <https://pubs.acs.org/doi/abs/10.1021/bm049306e>.
- Ahearne, M, Y Yang, and K-K Liu (2008). *Mechanical Characterisation of Hydrogels for Tissue Engineering Applications Hydrogels for Tissue Engineering*. Tech. rep. URL: https://www.oulu.fi/spareparts/ebook_topics_in_t_e_vol4/abstracts/ahearne.pdf.
- Rowley, Jon A., Gerard Madlambayan, and David J. Mooney (1999). "Alginate hydrogels as synthetic extracellular matrix materials". In: *Biomaterials* 20.1, pp. 45–53. ISSN: 01429612. DOI: [10.1016/S0142-9612\(98\)00107-0](https://doi.org/10.1016/S0142-9612(98)00107-0). arXiv: [NIHMS150003](https://arxiv.org/abs/NIHMS150003).
- Zhao, Xuanhe, Nathaniel Huebsch, David J Mooney, and Zhigang Suo (2010). "Stress-relaxation behavior in gels with ionic and covalent crosslinks". In: DOI: [10.1063/1.3343265](https://doi.org/10.1063/1.3343265). URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3069988/pdf/JAPIAU-000107-063509_1.pdf.
- Chaudhuri, Ovijit, Luo Gu, Darinka Klumpers, Max Darnell, Sidi A. Bencherif, James C. Weaver, Nathaniel Huebsch, Hong-pyo Lee, Evi Lippens, Georg N. Duda, and David J. Mooney (2016). "Hydrogels with tunable stress relaxation regulate stem cell fate and activity". In: *Nature Materials* 15.3, pp. 326–334. ISSN: 1476-1122. DOI: [10.1038/nmat4489](https://doi.org/10.1038/nmat4489). URL: <http://www.nature.com/articles/nmat4489>.
- Paluch, Ewa K, Celeste M Nelson, Nicolas Biais, Ben Fabry, Jens Moeller, Beth L Pruitt, Carina Wollnik, Galina Kudryasheva, Florian Rehfeldt, and Walter Federle (2015). "Mechanotransduction: use the force(s)." In: *BMC biology* 13, p. 47. ISSN: 1741-7007. DOI: [10.1186/s12915-015-0150-4](https://doi.org/10.1186/s12915-015-0150-4). URL: <http://www.ncbi.nlm.nih.gov/pubmed/26141078><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4491211>.
- Dupont, Sirio, Leonardo Morsut, Mariaceleste Aragona, Elena Enzo, Stefano Giullitti, Michelangelo Cordenonsi, Francesca Zanconato, Jimmy Le Diggabel, Mattia Forcato, Silvio Bicciato, Nicola Elvassore, and Stefano Piccolo (2011). "Role of YAP/TAZ in mechanotransduction". In: *Nature* 474. DOI: [10.1038/nature10137](https://doi.org/10.1038/nature10137). URL: <https://www.nature.com/articles/nature10137.pdf>.

- Jaalouk, Diana E. and Jan Lammerding (2009). "Mechanotransduction gone awry". In: *Nature Reviews Molecular Cell Biology* 10.1, pp. 63–73. ISSN: 1471-0072. DOI: [10.1038/nrm2597](https://doi.org/10.1038/nrm2597). URL: <http://www.nature.com/articles/nrm2597>.
- Goldmann, Wolfgang H. (2014). "Mechanosensation: A Basic Cellular Process". In: *Progress in Molecular Biology and Translational Science* 126, pp. 75–102. ISSN: 1877-1173. DOI: [10.1016/B978-0-12-394624-9.00004-X](https://doi.org/10.1016/B978-0-12-394624-9.00004-X). URL: <https://www.sciencedirect.com/science/article/pii/B978012394624900004X>.
- Engler, Adam J., Shamik Sen, H. Lee Sweeney, and Dennis E. Discher (2006). "Matrix Elasticity Directs Stem Cell Lineage Specification". In: *Cell* 126.4, pp. 677–689. ISSN: 0092-8674. DOI: [10.1016/J.CELL.2006.06.044](https://doi.org/10.1016/J.CELL.2006.06.044). URL: <https://www.sciencedirect.com/science/article/pii/S0092867406009615>.
- Darnell, Max, Luo Gu, and David Mooney (2018). "RNA-seq reveals diverse effects of substrate stiffness on mesenchymal stem cells." In: *Biomaterials* 181, pp. 182–188. ISSN: 1878-5905. DOI: [10.1016/j.biomaterials.2018.07.039](https://doi.org/10.1016/j.biomaterials.2018.07.039). URL: <https://linkinghub.elsevier.com/retrieve/pii/S0142961218305246><http://www.ncbi.nlm.nih.gov/pubmed/30086447><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6258186>.
- Darnell, Max, Alison O'Neil, Angelo Mao, Luo Gu, Lee L Rubin, and David J Mooney (2018). "Material microenvironmental properties couple to induce distinct transcriptional programs in mammalian stem cells." In: *Proceedings of the National Academy of Sciences of the United States of America* 115.36, E8368–E8377. ISSN: 1091-6490. DOI: [10.1073/pnas.1802568115](https://doi.org/10.1073/pnas.1802568115). URL: <http://www.ncbi.nlm.nih.gov/pubmed/30120125><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6130338>.

Chapter 2

Methods

2.1 Alginate Hydrogels System

2.1.1 Alginate Preparation

I1G, a high G content alginate with a high molecular weight (260 kPa), was purchased from KIMICA Corporation, and was prepared as has been described in previous paper (Rowley, Madlambayan, and Mooney, 1999). Also, high-MW I1G alginate was irradiated by a 3, 5 or 8 Mrad cobalt source to produce medium MW or low MW alginates. RGD-coupled alginate was prepared by coupling the peptide GGGGRGDSP (Peptide 2.0 Inc) using carbodiimide chemistry and 20 RGD peptides were coupled to one alginate chain on average (DS 20), based on the highest MW alginate. Then alginate was purified by dialysis (3500 MWCO) against deionized water containing Sodium Chloride for 3 days, treated with activated charcoal, sterile filtered, lyophilized until dried and then reconstituted in serum-free DMEM (ThermoFisher/Invitrogen).

2.1.2 Alginate Characterization

Molecular weights of alginates were analysed with Agilent 1200 series GPC equipped with miniDAWN TREOSTM light scattering (LS) detector, Agilent Chamstation software and ASTRA software. Samples were dissolved in 0.2 M NaNO₃ and 0.01 M NaH₂PO₄ buffer solution at a concentration of 1 mg ml⁻¹, and 200 µl of samples was injected. Alginates were separated through one PL aquagel-OH MIXED-H column with 0.5 ml min⁻¹ flow rate and were analysed by triple light scattering detector system. Agilent EasiVial PEG/PEO standards were used in calibration, and weight-average molecular weights (M_w) were used.

2.1.3 Alginate Hydrogel Fabrication

Calcium sulfate (CaSO₄) is used as a source for the release of gelling ions. After mixing with alginate, the mixture was disposed on a glass plate coated with Sigmacote[®] and cover with another coated glass plate. The thickness of the hydrogel was controlled by the thickness of glass slides on both sides.

2.1.4 Mechanical Characterization

The elastic modulus and stress relaxation properties of alginate hydrogels were measured by compression tests of the gel disks (15 mm in diameter, 2 mm thickness, equilibrated in serum-free DMEM for 24 h before testing) based on a previously published method (Zhao et al., 2010). The gel disks were compressed to 15% strain with a 2 mm min⁻¹ deformation rate and 100 Hz data acquisition rate using MTS Criterion[®] Series 40 Tensile Tester. The slope

of the stress-strain curve (first 5%-10% strain linear part) gives the elastic modulus. And then, the strain was kept constant, as the load was recorded over time. Half-time ($\tau_{1/2}$) is the quantification of stress relaxation properties, which is the time for the stress to relaxed to half its original value.

2.2 2D Single Cell Tracking

RGD-coupled alginate with high, medium and low MW was reconstituted in serum-free DMEM (ThermoFisher/Invitrogen) to 2.5% (w/v). Then alginate was mixed with different concentrations of CaSO_4 to form hydrogels with different mechanical properties. After gelling for 45 min, gels were punched to 15 mm disks and gel disks were equilibrated in serum-free DMEM for 24 h. Then gels were transferred to 12-well plate. Transwell holders were used to hold the gels at the bottom of the wells. 1.2 ml DMEM containing 10% FBS (Hyclone) and 1% Pen/Strep (ThermoFisher/Invitrogen) was added to the outside region of transwell holder and 0.8 ml media was added inside the transwell holder. Subsequently, 100 μl cell media containing 2×10^4 NIH/3T3-GFP cells (CELL BIOLABS, INC.) was added inside the transwell holder and the cells were cultures for 24 h in 37 °C incubator. The NIH/3T3-GFP cell movement was captured for up to 3 h by Nikon TE2000 microscope every 5 min. The average speed of 2D single cell migration was manually tracked by CellTracker (Piccinini, Kiss, and Horvath, 2016) software.

2.3 2D Wound Model

RGD-coupled alginate with high, medium and low MW was reconstituted in serum-free DMEM (ThermoFisher/Invitrogen) to 2.5% (w/v). Then alginate was mixed with different concentrations of CaSO_4 to form hydrogels with different mechanical properties. After gelling for 45 min, gels were punched to 15 mm disks and gel disks were equilibrated in serum-free DMEM for 24 h. Then gels were transferred to 12-well plate. Polylactic Acid (PLA) Transwell holders were printed by creality 3D[®] CR-10 printer. Then a cover glass coated with Sigmacote[®] was inserted inside a transwell holder to help to create empty wound area and also was used to hold the gels at the bottom of the wells. 1.2 ml DMEM containing 10% FBS (Hyclone) and 1% Pen/Strep (ThermoFisher/Invitrogen) was added to the outside region of transwell holder and 0.8 ml media was added inside the transwell holder. Subsequently, 100 μl cell media containing 3×10^5 NIH/3T3-GFP cells (CELL BIOLABS, INC.) was added to each side of the cover glass inside the transwell holder and the cells were cultured for 3 h in 37 °C incubator. Then the printed transwell holders with cover glasses were replaced with standard transwell holders. After the replacement, the NIH/3T3-GFP cells movement was captured by EVOS M5000 microscope at 0 h and 19 h time points. Wound area can be calculated by measure the cell-free area in captured images by Imagej software (Fig.2.1). The 2D population migration rate can be expressed as the percentage of area reduction or wound closure over time(Grada et al., 2017).

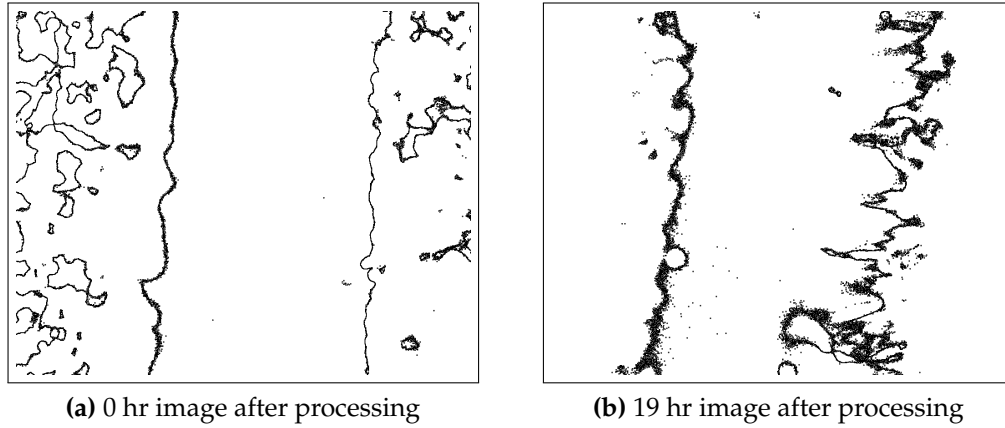


Figure 2.1: Examples processed images by Imagej software at 0 h and 19 h for 2D wound model. The edge of the wound area was precisely defined by the Imagej software.

2.4 3D Single Cell Tracking

RGD-coupled alginate with high, medium and low MW was reconstituted in serum-free DMEM (ThermoFisher/Invitrogen) to 3% (w/v). Then alginate was first slowly mixed with certain amount of NIH/3T3-GFP cells (CELL BIOLABS, INC.) media, and the alginate and cell mixture was mixed with different concentrations of CaSO_4 to form hydrogels with different mechanical properties. And the cell density in the final hydrogels was designed to be 10^6 ml^{-1} . Cells were cultured in DMEM containing 10% FBS (Hyclone) and 1% Pen/Strep (ThermoFisher/Invitrogen) for 7 days and the culture medium was changed every 2 days. At day 7, the NIH/3T3-GFP cell movement was captured for up to 6 h by Nikon TE2000 microscope every 10 min. The average speed of 3D single cell migration was tracked by CellTracker (Piccinini, Kiss, and Horvath, 2016) software semi-automatically.

2.5 3D Radial Cell Migration

RGD-coupled alginate with high, medium and low MW was reconstituted in serum-free DMEM (ThermoFisher/Invitrogen) to 3% (w/v). The NIH/3T3-GFP cell (CELL BIOLABS, INC.) spheroids were made by Aggrewell™ 800 24-well Plate and were designed to be 5000 cells/spheroid. Then alginate was first slowly mixed with certain amount of NIH/3T3-GFP cell spheroids, and the alginate and spheroids mixture was mixed with different concentrations of CaSO_4 to form hydrogels with different mechanical properties. Cell spheroids were cultured in DMEM containing 10% FBS (Hyclone) and 1% Pen/Strep (ThermoFisher/Invitrogen) for 14days and the culture medium was changed every 3 days. The growth of cell protrusion was captured by EVOS M5000 microscope every day up to 14days. And the average length of the five longest protrusions was measured by ImageJ software.

References

- Rowley, Jon A., Gerard Madlambayan, and David J. Mooney (1999). "Alginate hydrogels as synthetic extracellular matrix materials". In: *Biomaterials* 20.1, pp. 45–53. ISSN: 0142-9612. DOI: [10.1016/S0142-9612\(98\)00107-0](https://doi.org/10.1016/S0142-9612(98)00107-0). URL: <https://www.sciencedirect.com/science/article/pii/S0142961298001070>.
- Zhao, Xuanhe, Nathaniel Huebsch, David J. Mooney, and Zhigang Suo (2010). "Stress-relaxation behavior in gels with ionic and covalent crosslinks". In: *Journal of Applied Physics* 107.6, p. 063509. ISSN: 0021-8979. DOI: [10.1063/1.3343265](https://doi.org/10.1063/1.3343265). URL: <http://aip.scitation.org/doi/10.1063/1.3343265>.
- Piccinini, Filippo, Alexa Kiss, and Peter Horvath (2016). "CellTracker (not only) for dummies". In: *Bioinformatics* 32.6, pp. 955–957. ISSN: 1367-4803. DOI: [10.1093/bioinformatics/btv686](https://doi.org/10.1093/bioinformatics/btv686). URL: <https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btv686>.
- Grada, Ayman, Marta Otero-Vinas, Francisco Prieto-Castrillo, Zaidal Obagi, and Vincent Falanga (2017). "Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay". In: *Journal of Investigative Dermatology* 137.2, e11–e16. ISSN: 15231747. DOI: [10.1016/j.jid.2016.11.020](https://doi.org/10.1016/j.jid.2016.11.020). URL: <http://dx.doi.org/10.1016/j.jid.2016.11.020>.

Chapter 3

Results

3.1 Alginate Hydrogels System

First, we developed an alginate hydrogels system with a wide range of stress relaxation rates, but with similar elastic modulus based on recent works (Chaudhuri et al., 2016). We used different molecular weight alginate, from 260 kPa to 27 kPa (Table 3.1), combined with different crosslink densities of calcium (Table 3.2). According to the results, the decrease in the elastic modulus due to decreased alginate molecular weight could be compensated by increasing crosslink density. The three targets of stiffness were 7 kPa, 12 kPa and 17 kPa (Fig. 3.1, 3.2 and 3.3). Mrad (mega-radiation absorbed dose) is one type of measure unit for expressing the absorption of ionizing radiation into any medium. So larger the number of Mrad, smaller the molecular weight of the alginates.

The half time ($\tau_{1/2}$), which is the time for the initial stress to be relaxed to half its value, for the alginate hydrogels was modulated from 2 h to 1 min

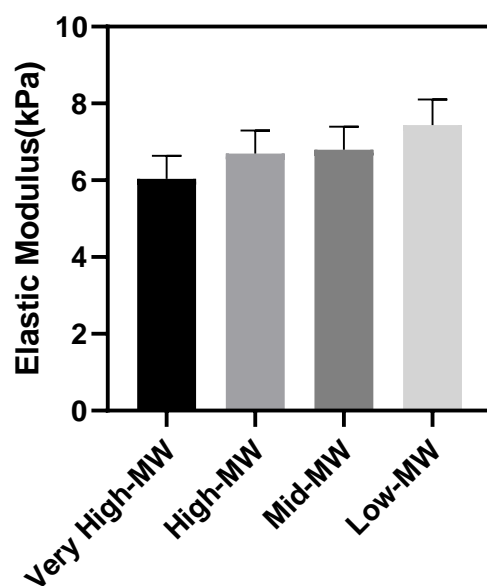


Figure 3.1: Elastic modulus measurements of gels targeting for 7kPa. Elastic modulus differences between four groups are not significant.

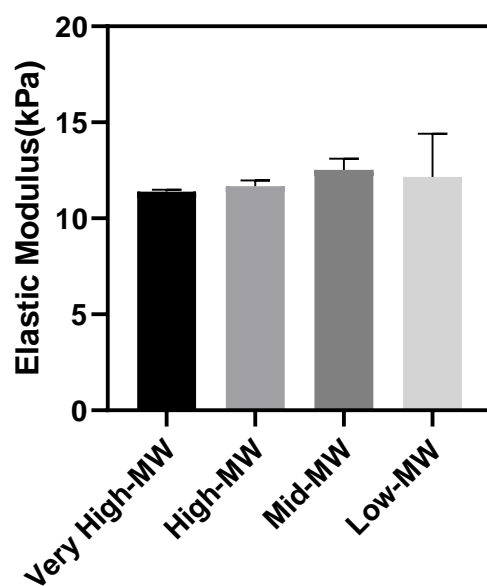


Figure 3.2: Elastic modulus measurements of gels targeting for 12kPa. Elastic modulus differences between four groups are not significant.

Alginate Types	Molecular Weight
I1G-Original (Very High-MW)	260kDa
I1G-3 Mrad (High-MW)	60kDa
I1G-5 Mrad (Mid-MW)	36kDa
I1G-8 Mrad (Low-MW)	27kDa

Table 3.1: Molecular Weight for four types of alginates used in experiments. Based on the results from GPC, the MW range of the alginates is from 260kPa to 27kPa.

(Fig.3.4, 3.5, 3.6 and Table 3.2), while keeping the alginate polymer concentration and the elastic modulus constant. Shorter the half time ($\tau_{1/2}$), faster the stress relaxation rate. Based on the results, the half time ($\tau_{1/2}$) decreased significantly with the decrease of MW while holding the elastic modulus constant, which showed the stress relaxation rate increased significantly when the MW of alginate decreased.

By using this alginate hydrogels system, we were able to control the matrix stress relaxation independent of the elastic modulus and polymer concentration and exclusively studied the influence of stress relaxation on cell behaviours, such as cell migration.

3.2 2D Single Cell Tracking

With this set of materials, the influences of stress relaxation rates on single cell migration in 2D culture was investigated. NIH/3T3-GFP cells were cultured on the surface of RGD-coupled alginate gels with different stress relaxation rates but with similar elastic modulus at around 7kPa and 17kPa, separately. Based on the results, the rates of substrate stress relaxation had significant effects on 2D single cell migration average speed. Basically, the 2D single

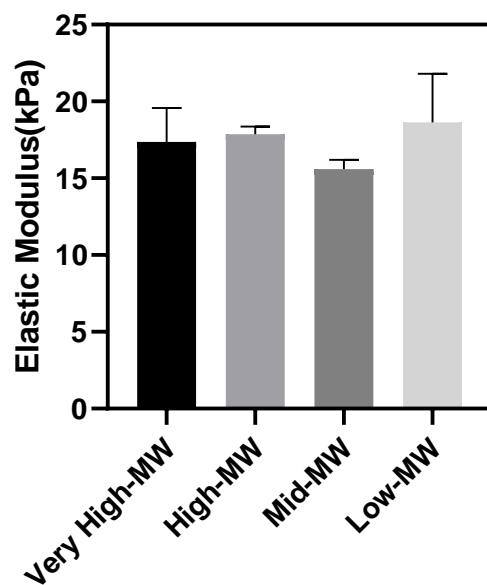


Figure 3.3: Elastic modulus measurements of gels targeting for 17kPa. Elastic modulus differences between four groups are not significant.

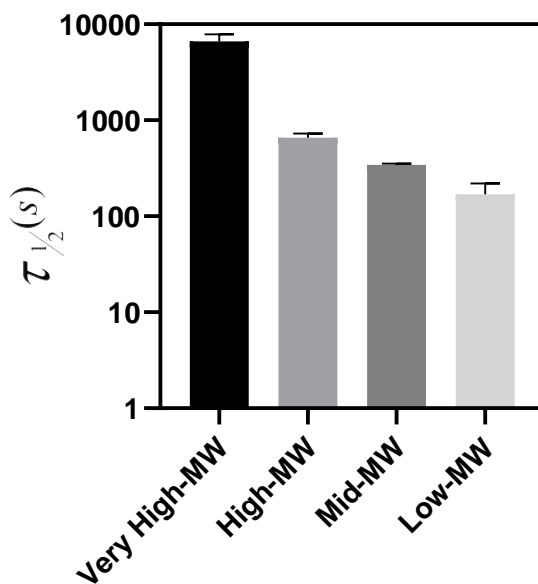


Figure 3.4: The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 7kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.

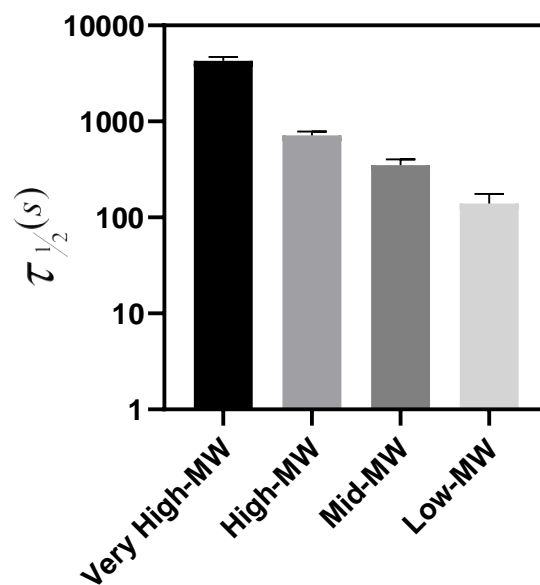


Figure 3.5: The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 12kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.

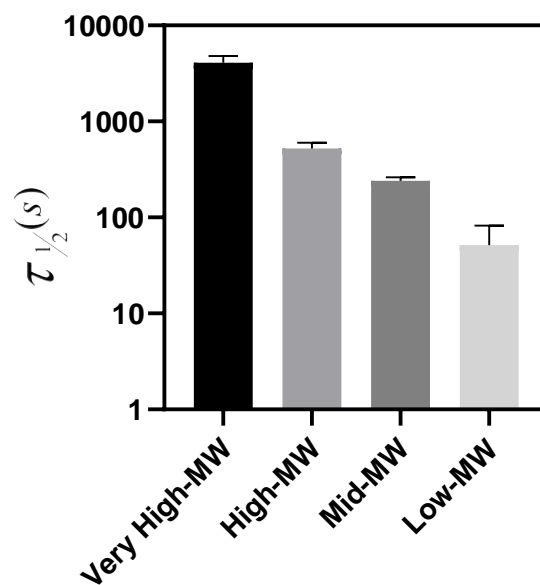


Figure 3.6: The time for the initial stress to be relaxed to half its value, $\tau_{1/2}$, from stress relaxation tests for 17kPa conditions. The timescale of stress relaxation for gels composed with different molecular weight decreases significantly.

Elastic modulus	Alginate Types	Ca ²⁺ Final Concentration in Gels (mmol)	Average $\tau_{1/2}$ (s)
7 kPa	I1G-original	12.2	6620
	I1G-3 Mrad	26.9	660
	I1G-5 Mrad	37.8	340
	I1G-8 Mrad	47.6	170
12 kPa	I1G-original	18.3	4290
	I1G-3 Mrad	34.2	720
	I1G-5 Mrad	42.7	300
	I1G-8 Mrad	53.7	140
17 kPa	I1G-original	25.6	4100
	I1G-3 Mrad	41.5	520
	I1G-5 Mrad	46.3	240
	I1G-8 Mrad	57.3	50

Table 3.2: Designed Ca²⁺ concentration in final gels and half time ($\tau_{1/2}$) for different hydrogels. Combined alginates with appropriate MW and Ca²⁺ concentration were able to form alginate hydrogels with different stress relaxation properties but similar elastic modulus.

cell average migration speed first increased, then decreased with the increase of the relaxation rates (Fig.3.7 and Table 3.3). And this phenomenon was more obvious in stiffer gel conditions (17 kPa). Also, in general, 2D single cell migration rate was found to increase with the decrease of elastic modulus of the substrates (Fig.3.7 and Table 3.3), which was corresponding to previous studies (Shukla et al., 2016; Lo et al., 2000; Vincent et al., 2013).

3.3 2D Wound Model

By using the designed setting of transwell holders and cover glasses, a regular and consistent "wound" area or gap was created on soft hydrogel surface without altering the curvature or roughness of the hydrogel surface (Fig.3.8a), which was proved to have influences on the cell migration rate (He and Jiang,

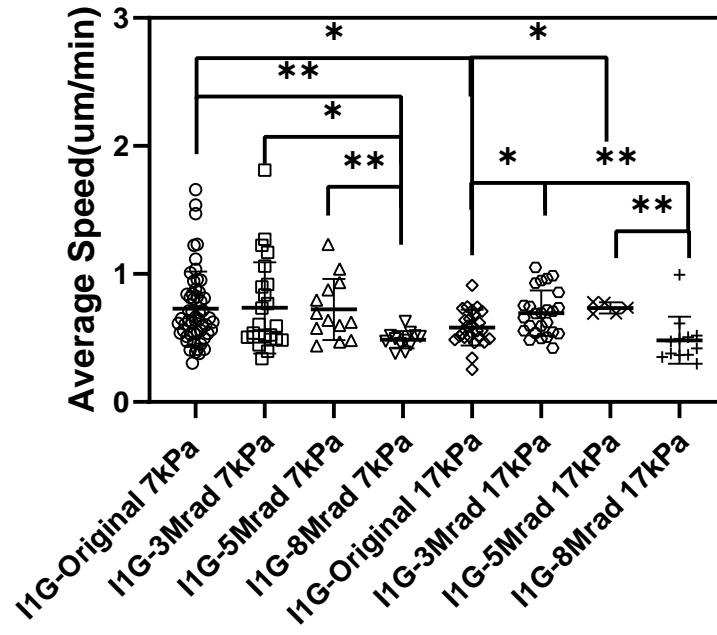


Figure 3.7: Quantification of average speed for 2D single cell migration with different mechanical properties. The average speed of 2D single cell migration increased first then decreased with the increase of stress relaxation rate or decreasing of half time $\tau_{1/2}$. *indicates $p < 0.05$ (Student's t-test), **indicates $p < 0.01$ (Student's t-test).

Elastic modulus	Alginate Types	Average Speed($\mu\text{m}/\text{min}$)
7 kPa	I1G-original	0.728
	I1G-3 Mrad	0.735
	I1G-5 Mrad	0.723
	I1G-8 Mrad	0.486
17 kPa	I1G-original	0.581
	I1G-3 Mrad	0.694
	I1G-5 Mrad	0.735
	I1G-8 Mrad	0.482

Table 3.3: Quantification of average speed for 2D single cell migration with different mechanical properties. The average speed of 2D single cell migration increased first then decreased with the increase of stress relaxation rates.

2017; Pieuchot et al., 2018). After culturing NIH/3T3-GFP cells on the surface of alginate hydrogels for 19 hours, part of the empty gap was closed by the cells (Fig. 3.8b). We only had the results for I1G-Original 17 kPa, I1G-3 Mrad 17 kPa and I1G-Original 7 kPa conditions, due to the limited time. But the preliminary conclusions are: the percentage of wound closure after 19h increased with the decreasing of elastic modulus of hydrogels and also increased with the increase of stress relaxation rate, which means the 2D collective cell migration rate increases with softer gels and faster stress relaxation (Table 3.4).

Elastic modulus	Alginate Types	Wound Closure% after 19h
17 kPa	I1G-original	3.9
	I1G-3 Mrad	29.5
7 kPa	I1G-original	60.3

Table 3.4: 2D Wound Model. The percentage of wound closure after 19h increased with the decreasing of elastic modulus of alginate hydrogels and also increased with the increase of stress relaxation rate.

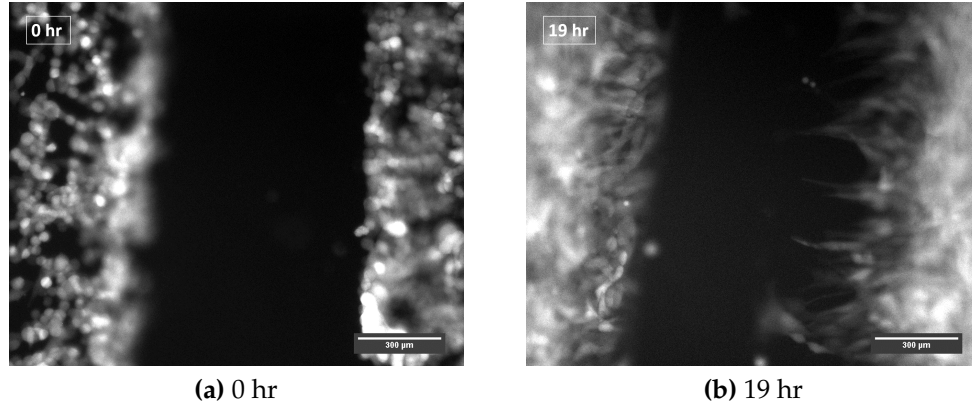


Figure 3.8: Examples images at 0 h and 19 h for 2D wound model. (a) A regular and consistent gap was created on soft hydrogel surface without altering the soft hydrogel substrate by the method we developed. (b) After 19 hours, part of the gap was closed by cells.

3.4 3D Single Cell Tracking

NIH/3T3-GFP cells were encapsulated within RGD-coupled alginate hydrogels with varied stress relaxation rates and with constant elastic modulus of ~ 7 kPa, and were cultured for 7 days. Based on the results, the 3D single cell migration rate was enhanced by increasing the stress relaxation rate, while holding the elastic modulus constant and keeping the final cell concentration at 10^6 ml^{-1} (Fig.3.9 and Table 3.5).

Elastic modulus	Alginate Types	Average Speed($\mu\text{m}/\text{min}$)
7 kPa	I1G-original	0.049
	I1G-8 Mrad	0.074

Table 3.5: Average speed for 3D single cell migration. 3D single cell migration rate was enhanced by increasing the stress relaxation rate.

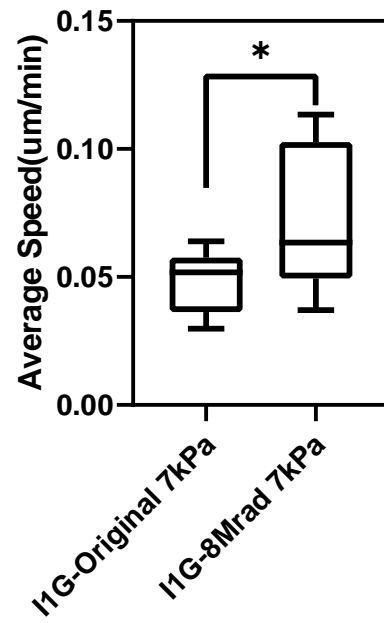


Figure 3.9: Quantification of average speed for 3D single cell migration with different stress relaxation rate. *indicates $p < 0.05$ (Student's t-test). When holding the elastic modulus constant and final cell concentration, the average speed of 3D single cell migration was suppressed with the increase of stress relaxation rate or decreasing of half time $\tau_{1/2}$. *indicates $p < 0.05$ (Student's t-test).

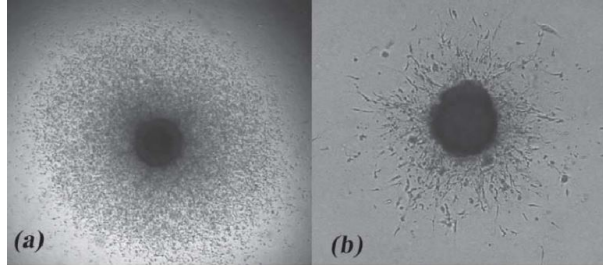
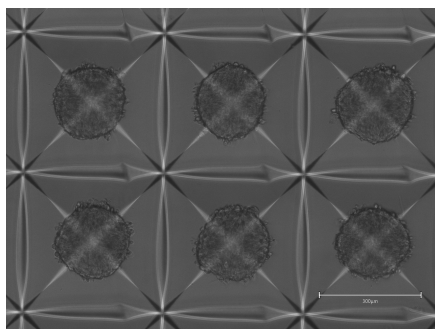


Figure 3.10: Example images of two 3D radial cell migration patterns. (a) Radially symmetric migration of individual cells; (b) Formation of protrusions (Khain and Sander, 2006).

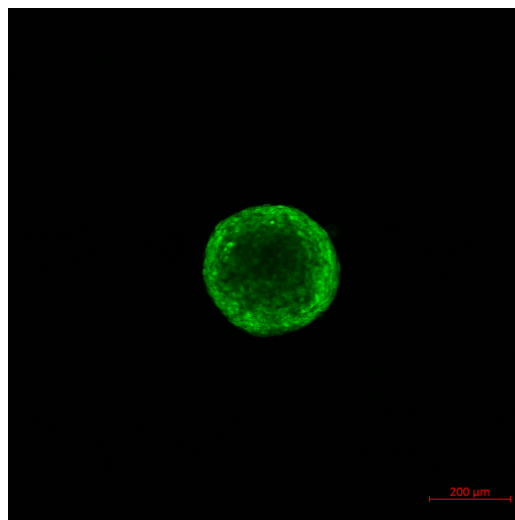
3.5 3D Radial Cell Migration

NIH/3T3-GFP cell spheroids, with regular shape and tunable size, were made by using Aggrewell™ 800 24-well Plate (Fig.3.11). Cell spheroids were encapsulated within RGD-coupled alginate hydrogels with varying stress relaxation properties. There are usually two different 3D radial migration patterns (Kim et al., 2009; Stein et al., 2007; Khain and Sander, 2006) (Fig.3.10): one exhibits radially symmetric migration of individual cells, with a faster speed; for another pattern, cells form protrusions moving outward, with a slower speed. The 3D radial migration pattern of NIH/3T3-GFP cells encapsulated in alginate hydrogels was protrusions pattern (Fig.3.12). Fig.3.13 shows the growth of protrusions with time in fast stress relaxation gels: the protrusions were already obvious at day 3 and kept growing even after 9 days.

Based on the results, 3D radial migration was suppressed within hydrogels with long timescales for stress relaxation ($\tau_{1/2} > 4$ min) and with high elastic modulus (Fig.3.14). There was only obvious 3D radial cell migration when cell spheroids were encapsulated within alginate hydrogels with fast relaxation rate (I1G-8 Mrad) (Fig.3.14). And higher the elastic modulus of alginate



(a) Cell spheroids inside TM 800



(b) Cell spheroids

Figure 3.11: Examples images of cell spheroids. (a) Regular and designed size of cell spheroids were made by AggrewellTM 800 24-well Plate. (b) Confocal images of one cell spheroid.

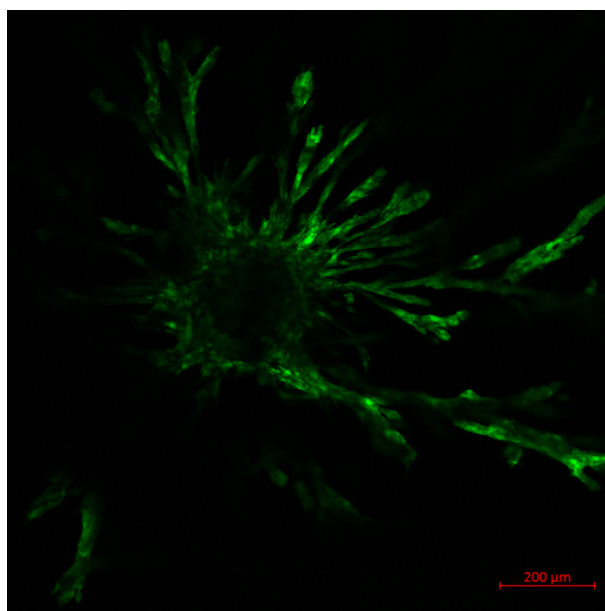


Figure 3.12: Example confocal image of 3D radial cell migration. NIH/3T3-GFP cell spheroids showed 3D radial protrusion pattern when encapsulated in alginate hydrogels.

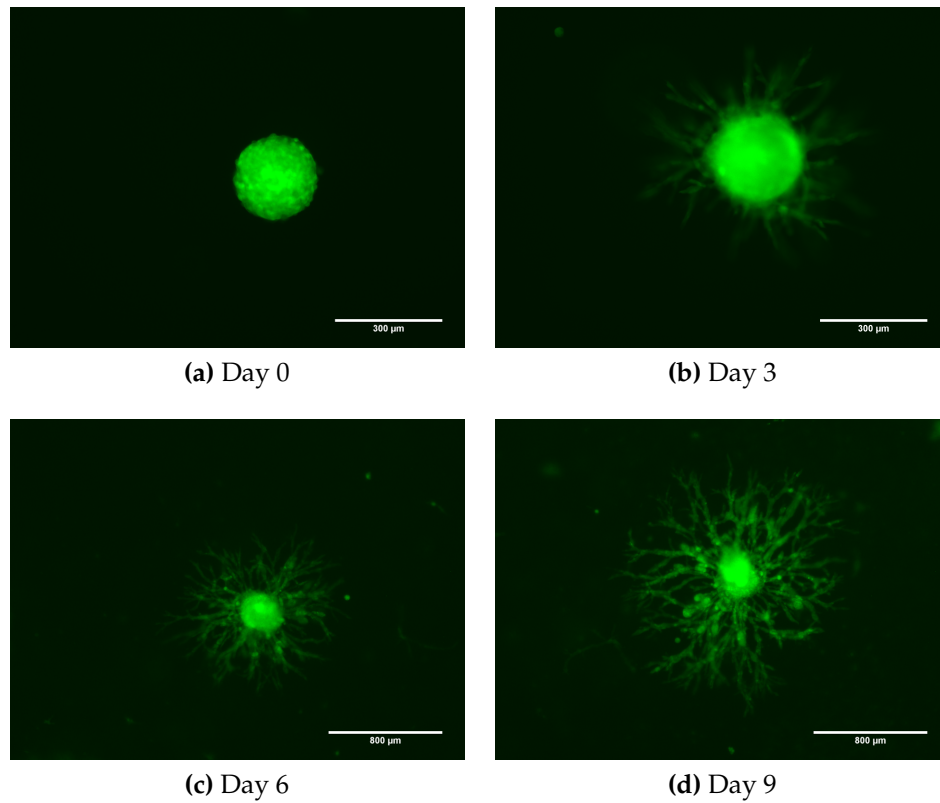


Figure 3.13: Examples images showed the growth of spheroids protrusions with time. There were obvious 3D radial protrusion migration phenomenon when cell spheroids were encapsulated in hydrogels (I1G-8 Mrad 7 kPa) with fast relaxation rate.

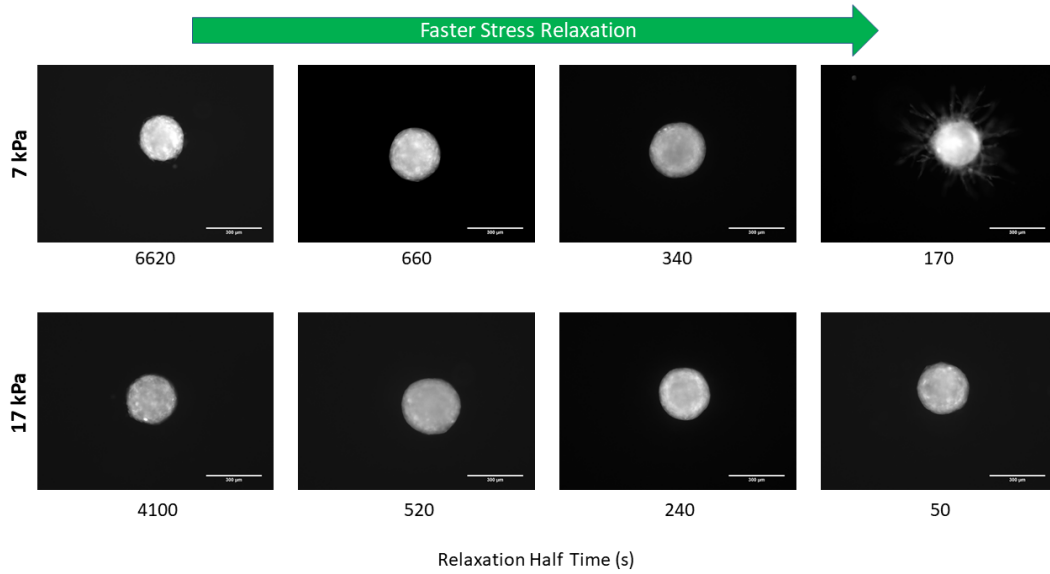


Figure 3.14: Representative images of NIH/3T3-GFP spheroids encapsulated within alginate gels with the indicated relaxation time $\tau_{1/2}$ and two elastic modulus (7 kPa and 17 kPa). Images were taken after three days in culture. Scalebar is 300 μm . There was only obvious protrusion migration in fast stress relaxation conditions (I1G - 8 Mrad).

hydrogels, shorter the average length of the longest protrusions and lower the densities of the protrusions (Fig.3.15, 3.16 and Table 3.6). Also, the morphology of the protrusions were different between the stiffer gels and softer gels: when grew within the softer gels, like 7 kPa conditions, the protrusions were thinner and with a larger number of branches; when grew within the stiffer gels, like 12 kPa and 17 kPa conditions, the protrusions were thicker and with a smaller number of branches. From day1, there was significant differences of the average length of longest protrusion between 7 kPa and 12 kPa, 17 kPa conditions. And From day4, it has started to have significant differences of the average length of longest protrusion between 12 kPa and 17 kPa conditions.

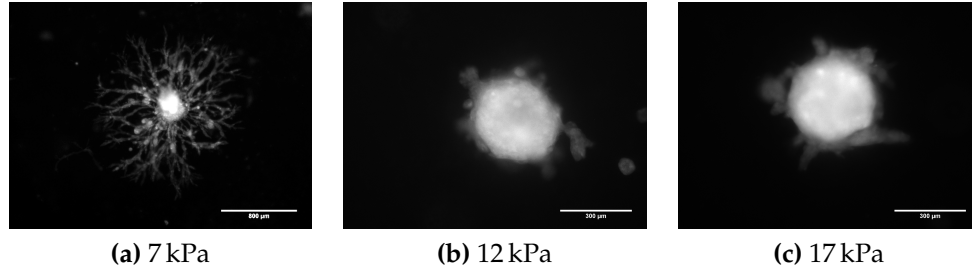


Figure 3.15: Examples images of 3D radial migration phenomenon for 3 different stiffness hydrogels conditions with fast stress relaxation properties. (a)Alginate hydrogels with 7 kPa elastic modulus had the longest and densest protrusions. (b)Alginate hydrogels with 12 kPa elastic modulus had the medium length and medium density of protrusions. (c)Alginate hydrogels with 17 kPa elastic modulus had the shortest and low density of protrusions.

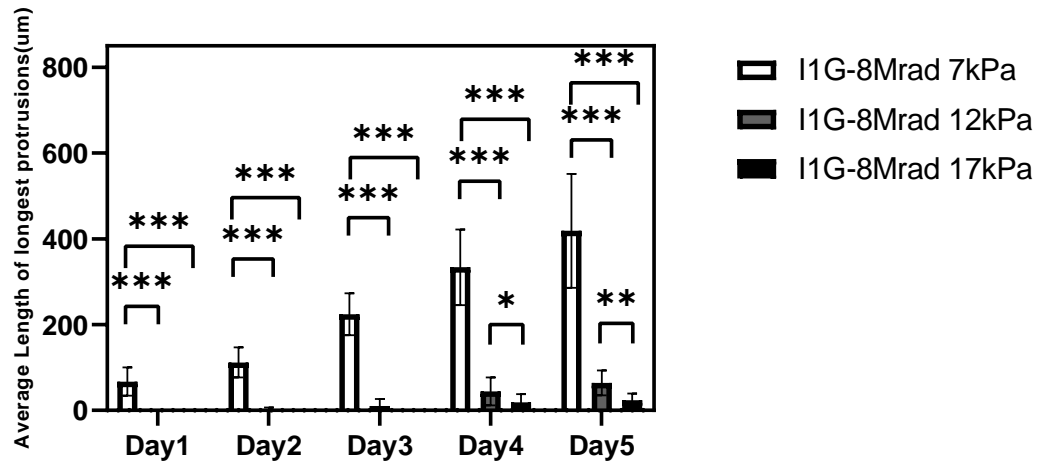


Figure 3.16: Quantification of the average length of longest protrusions for 3 different stiffness hydrogels conditions with fast stress relaxation rates. Stiffer the alginate hydrogels, shorter the average length of the longest protrusions.*indicates $p < 0.05$ (Student's t-test), **indicates $p < 0.01$ (Student's t-test), ***indicates $p < 0.001$ (Student's t-test).

Elastic modulus	Average Length of Longest protrusions (μm)				
	Day 1	Day 2	Day 3	Day 4	Day 5
7 kPa	67.1	111.9	224.0	333.7	418.7
12 kPa	0.0	1.7	10.3	44.1	64.3
17 kPa	0	0	0	19.1	24.0

Table 3.6: Average length of longest protrusions for 3 different stiffness hydrogels conditions with fast stress relaxation rates. Higher the elastic modulus of alginate hydrogels, shorter the average length of the longest protrusions.

References

- Chaudhuri, Ovijit, Luo Gu, Darinka Klumpers, Max Darnell, Sidi A. Bencherif, James C. Weaver, Nathaniel Huebsch, Hong-pyo Lee, Evi Lippens, Georg N. Duda, and David J. Mooney (2016). "Hydrogels with tunable stress relaxation regulate stem cell fate and activity". In: *Nature Materials* 15.3, pp. 326–334. ISSN: 1476-1122. DOI: [10.1038/nmat4489](https://doi.org/10.1038/nmat4489). URL: <http://www.nature.com/articles/nmat4489>.
- Shukla, V. C., N. Higueta-Castro, P. Nana-Sinkam, and S. N. Ghadiali (2016). "Substrate stiffness modulates lung cancer cell migration but not epithelial to mesenchymal transition". In: *Journal of Biomedical Materials Research Part A* 104.5, pp. 1182–1193. ISSN: 15493296. DOI: [10.1002/jbm.a.35655](https://doi.org/10.1002/jbm.a.35655). URL: <http://www.ncbi.nlm.nih.gov/pubmed/26779779><http://doi.wiley.com/10.1002/jbm.a.35655>.
- Lo, Chun-Min, Hong-Bei Wang, Micah Dembo, and Yu-li Wang (2000). "Cell Movement Is Guided by the Rigidity of the Substrate". In: *Biophysical Journal* 79.1, pp. 144–152. ISSN: 0006-3495. DOI: [10.1016/S0006-3495\(00\)76279-5](https://doi.org/10.1016/S0006-3495(00)76279-5). URL: <https://www.sciencedirect.com/science/article/pii/S0006349500762795>.
- Vincent, Ludovic G., Yu Suk Choi, Baldomero Alonso-Latorre, Juan C. del Álamo, and Adam J. Engler (2013). "Mesenchymal stem cell durotaxis depends on substrate stiffness gradient strength". In: *Biotechnology Journal* 8.4, pp. 472–484. ISSN: 18606768. DOI: [10.1002/biot.201200205](https://doi.org/10.1002/biot.201200205). URL: <http://doi.wiley.com/10.1002/biot.201200205>.
- He, Xiuxiu and Yi Jiang (2017). "Substrate curvature regulates cell migration". In: *Physical Biology* 14.3, p. 035006. ISSN: 1478-3975. DOI: [10.1088/1478-3975/aa6f8e](https://doi.org/10.1088/1478-3975/aa6f8e). URL: <http://www.ncbi.nlm.nih.gov/pubmed/28535145><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5572487><http://stacks.iop.org/1478-3975/14/i=3/a=035006?key=crossref.88ed625e87aadead54959949fc9abc8b>.

- Pieuchot, Laurent, Julie Marteau, Alain Guignandon, Thomas Dos Santos, Isabelle Brigaud, Pierre-François Chauvy, Thomas Cloatre, Arnaud Ponche, Tatiana Petithory, Pablo Rougerie, Maxime Vassaux, Jean-Louis Milan, Nayana Tusamda Wakhloo, Arnaud Spangenberg, Maxence Bigerelle, and Karine Anselme (2018). "Curvotaxis directs cell migration through cell-scale curvature landscapes". In: *Nature Communications* 9.1, p. 3995. ISSN: 2041-1723. DOI: [10.1038/s41467-018-06494-6](https://doi.org/10.1038/s41467-018-06494-6). URL: <http://www.ncbi.nlm.nih.gov/pubmed/30266986><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6162274><http://www.nature.com/articles/s41467-018-06494-6>.
- Kim, Yangjin, Sean Lawler, Michal O Nowicki, E Antonio Chiocca, and Avner Friedman (2009). "A mathematical model for pattern formation of glioma cells outside the tumor spheroid core". In: DOI: [10.1016/j.jtbi.2009.06.025](https://doi.org/10.1016/j.jtbi.2009.06.025). URL: www.elsevier.com/locate/yjtbi.
- Stein, Andrew M., Tim Demuth, David Mobley, Michael Berens, and Leonard M. Sander (2007). "A Mathematical Model of Glioblastoma Tumor Spheroid Invasion in a Three-Dimensional In Vitro Experiment". In: *Biophysical Journal* 92.1, pp. 356–365. ISSN: 0006-3495. DOI: [10.1529/BiophysJ.106.093468](https://doi.org/10.1529/BiophysJ.106.093468). URL: <https://www.sciencedirect.com/science/article/pii/S0006349507708330>.
- Khain, Evgeniy and Leonard M. Sander (2006). "Dynamics and Pattern Formation in Invasive Tumor Growth". In: *Physical Review Letters* 96.18, p. 188103. ISSN: 0031-9007. DOI: [10.1103/PhysRevLett.96.188103](https://doi.org/10.1103/PhysRevLett.96.188103). URL: <https://link.aps.org/doi/10.1103/PhysRevLett.96.188103>.

Chapter 4

Discussion

Cell migration plays a central role in various of biological processes including embryonic development, wound healing, immune responses and cancer metastasis (Fallis, [2013](#); Lauffenburger and Horwitz, [1996](#)). Cells can migrate in response to external signals, including chemical signals and mechanical signals (Fallis, [2013](#)). An understanding of mechanisms of cell migration may help with the development of novel therapeutic strategies for controlling cell migration properties.

There are different ways to classify the types of cell migration. Based on different cell-type-specific patterns, cell migration can be classified into single cell models and collective migration models (Decaestecker et al., [2006](#)). The distinction of collective migration from single cell migration is that during migration cells keep connection and maintain cell-cell junctions, as well as the migration patterns are basically different from single cell migration (Lintz, Muñoz, and Reinhart-King, [2017](#)). Single cell migration models can be used to study biological processes involving the migration of single cell in the context of inflammatory, immune reaction and tumor invasions (Decaestecker et al.,

2006). And *in vivo*, collective cell migration usually happens during wound healing, embryogenesis and tumor invasions (Friedl and Gilmour, 2009). For examples, when tumor cells enter into vasculatures, cells can circulate as single cells or form a cluster known as circulating tumor microemboli (CTM) (Duda et al., 2010).

Also based on dimension of the system, cell migration can be classified into 2D migration and 3D migration (Decaestecker et al., 2006). 2D models have been traditionally used to study cell migration properties and are much easier to conduct compared to 3D models. Despite its simplicity, there are certain biologic processes *in vivo* that represent 2D migration models. For instance, T cells migrate on 2D endothelial cell surfaces through the body in order to respond to infection cues and conduct immunologic functions (Horssen and Hagen, 2011). Nevertheless, previous studies showed that cells cultured in 3D matrix have different shapes, profiles of gene expression, level of proliferation and capacities for multicellular organization with cells cultured on 2D matrix (Vernon and Gooden, 2002). So, it is necessary to study both 2D and 3D cell migration models in order to fully understand the influence of stress relaxation on cell migration.

Four models of cell migrations were studied in the field: 2D single cell migration model, 2D collective migration model, 3D single cell migration model and 3D collective migration model. To study these migration models, a number of different *in vitro* methodologies are available to characterize cell migration properties (Decaestecker et al., 2006; Fallis, 2013). In general, for 2D single cell migration models, there are colloidal gold migration assay, single

cell tracking assay and cell chemotaxis assay; for 2D collective migration models, there are microcarrier bead assay, monolayer wound model and ring assays; for 3D single cell migration models, there is 3D single cell tracking; for 3D collective migration models, there are transwell cell culture chamber, collagen invasion assay, invasion in an organ culture and 3D radial cell migration assays (Decaestecker et al., 2006). In our experiments, 2D single cell tracking, 2D wound model, 3D single cell tracking and 3D radial assay were studied.

4.1 Alginate Hydrogels System

In the past few decades, researchers did extensive studies about how the stiffness of substrate regulate cell behaviours, such as cell spreading (Pelham, And, and Wang, 1998; Engler et al., 2004), cell migration (Lo et al., 2000), cell proliferation (Wang, Tsai, and Voelcker, 2012) and cell differentiation (Deroanne, Lapiere, and Nusgens, 2001; Flanagan et al., 2006; Engler et al., 2006a; Huebsch et al., 2010). And hydrogels systems have been developed and engineered to mimic native extracellular matrix (ECM) stiffness in order to investigate cell behaviours (Discher, Mooney, and Zandstra, 2009). However, ECM and various tissues organ, such as brain, adipose tissue or coagulated bone marrow all exhibit viscoelastic properties (Chaudhuri et al., 2016). Recently, researchers have found that substrate viscoelasticity has impacts on various cell behaviors (Chaudhuri et al., 2015; Cameron, Frith, and Cooper-White, 2011; Cameron et al., 2014). Therefore, based on recent works (Chaudhuri et al., 2016), we chose ionic cross-linked alginate hydrogels system as substrates for cell culture. We could modulate the nano-structure of gels to develop a materials system with

varying stress relaxation rates, while keeping elastic moduli and polymer concentrations constant. By combining different molecular weight polymers with different crosslink densities of calcium, the stress relaxation properties of alginates hydrogels could be tuned due to the varied connectivity and chain mobility (Graessley, 1982) in the network. Decrease of elastic modulus owing to decreased alginate molecular weight could be compensated for by increasing crosslink density. Here, the alginate hydrogels system was designed to have three stiffness groups (Fig. 3.1, 3.2 and 3.3): 7 kPa, 12 kPa and 17 kPa, within the range of the stiffness of ECM and tissues (Hadden et al., 2017), and each stiffness group had 4 different stress relaxation rates based on the MW (Table 3.1 and 3.2).

Also, alginates do not present intrinsic integrin-binding sites and are not able to interact with cells, but cell adhesion could be promoted by covalently modified alginates chain with RGD peptide (Rowley, Madlambayan, and Mooney, 1999). The ligand densities of RGD peptide could be adjusted based on the concentration of peptide available for reaction (Rowley, Madlambayan, and Mooney, 1999) and according to previous studies, cell spreading and cell proliferation were enhanced by high density of RGD peptide (Rowley, Madlambayan, and Mooney, 1999). Therefore, in our experiments, we only used alginates coupled with high RGD peptide density (DS 20) for cell experiments to enhance the cell migration activities.

Here, the alginate hydrogels system was developed to control substrate stress relaxation rates, independent of the elastic modulus and polymer concentration. In order to basically understand the influence of stress relaxation

to cell migration, we experimented some extreme conditions of mechanical properties to compare the fastest relaxation rate with slowest relaxation rate.

4.2 2D Single Cell Tracking

The migration of single cells needs the asymmetrical organization of cellular activities and many different types of cell become polarized with a front and rear asymmetry, which results in random migration (Fallis, 2013). Single cell models allow the cell migration to be distinguished from cell proliferation and single cell tracking is one of the commonly used assay to investigate single cell migration properties (Decaestecker et al., 2006; Ascione et al., 2016). Also, 2D single cell models are suitable to investigate the biologic processes that involve single cells migrating on 2D surface, such as T cells migrating on endothelial cell surfaces of circulatory system (Horssen and Hagen, 2011).

Single cell tracking usually involves time-lapse microscopy technique that automatically recording movements of living cells with constant time interval. The (t, X, Y) coordinates of each cell are tracked to establish cell trajectories. Then features that represent the cell migration can be qualified and quantified, such as speed, direction, distance and duration of the cell migration.

Previous studies showed that focal-adhesion and cytoskeleton could be influenced by a wide range variation in substrates stiffness (Cukierman et al., 2001; Bershadsky, Balaban, and Geiger, 2003), and cells exhibited different cell behaviors when cultured in hydrogels with the elastic modulus of 1-10 kPa or 11-30 kPa (Huebsch et al., 2010). Therefore, in our experiments, cells were cultured on the surface of alginate hydrogels with two different stiffness (7 kPa

and 17 kPa) within the stiffness range of normal muscle tissue (Engler et al., 2006b). First, NIH/3T3-GFP cells were cultured on the surface of alginate hydrogels with different elastic modulus and different stress relaxation rates for 24 hours before imaging, in order to let cells attach to the gels and fully spread on the surface. And the cell density on the surface was maintained at the low cell setting density ($2 \times 10^4 \text{ cm}^{-2}$) on the surface of alginate hydrogels to get enough number of single cell on the surface.

Due to the insufficient data in some groups (like I1G-5 Mrad 17 kPa), we were only able to make primary conclusions here. The primary conclusions were that the 2D single cell migration speed was suppressed, when cells migrated on the surface of gels with either long timescales for stress relaxation ($\tau_{1/2} > 1h$) or short timescales for stress relaxation ($\tau_{1/2} < 1 \text{ min}$). So with the increase of stress relaxation rates, the 2D single cell average migration speed first increased and then decreased, which could be represented as the inverted-U shaped relationship (Fig. 3.7 and Table 3.3). However, the cell passage number varied a lot between different hydrogels conditions, which might be another possible reason for the results of 2D single cell migration study. Therefore, we need more data to confirm the results.

Based on the previous studies of the mechanisms of cell migration, there are a lot of factors that have influences on the cell migration processes, like the formation of polarized networks of actin filaments and micro-tubules, asymmetric distribution of signaling molecules, the formation of focal adhesions and the activity of matrix-degrading proteases (Decaestecker et al., 2006; Sugawara et al., 2016; De Pascalis and Etienne-Manneville, 2017). Also,

previous studies on the influence of stiffness to cell migration shows the focal adhesions are more stable and larger on stiffer gels and are more dynamic and smaller on softer gels (De Pascalis and Etienne-Manneville, 2017). Specifically, focal adhesions promoted the cell adhesions and the signaling between the substrates and cells, however the relationship between the size and number of focal adhesion clusters and cell migration speed is not linear (Kim and Wirtz, 2013b; Kim and Wirtz, 2013a). Even though we can not use the shape and number of focal adhesions to forecast cell migration, recent works showed that both speed and persist distance of 2D cell migration first increased, then decrease while increasing the size of focal adhesions (Wu, Gilkes, and Wirtz, 2018). Therefore, the possible reasons for our results of the 2D single cell migration studies could be that the size of focal adhesions increases with the increase of stress relaxation rate. More experiments are needed to understand the mechanisms of the impact of stress relaxation to 2D single cell migration.

4.3 2D Wound Model

There are four main stages in wound healing: coagulation, inflammation, migration-proliferation and remodeling (Falanga, 2005). And fibroblast is a type of cells that synthesize ECM, collagen and plays important role in normal wound healing process (P., 2013). The NIH/3T3 cell line is one of the the standard fibroblast cell lines, which is the main reason why we used NIH/3T3 cells in the 2D wound model.

In vitro, scratch or wound of a cell monolayer can induce the synchronized movement of sheets of cells, which produce the healing processes of scratch

or wound (Fallis, 2013). Collective cell migration allows cells to interact with their neighbors providing additional directional cues, which is different from single cell migration model.

The wound healing model is among the most commonly used assays to investigate cell collective migration *in vitro* and is one of the earliest developed methods for cell migration study ("[Wound-Healing Assay](#)"; Grada et al., 2017; Fallis, 2013). Wound healing model is able to mimic cell migration during wound healing process *in vivo*. Basically, there are three steps of wound-healing assay. First, create an empty "wound" area in a cell mono-layer. Then take images from the beginning and with constant time interval during the migration process. And compare the images to quantify the collective cell migration rate of cells. The critical step for wound-healing assay is the creation of "wound" or gap. Usually for hard substrate, gap can be created by an object such as pipette tips, syringe needles or even electrical currents ("[Wound-Healing Assay](#)"; Grada et al., 2017). However, for soft substrates, like hydrogels, it is not suitable to create the gap using the objects like pipette tips, since it can damage the surface of soft substrates and the curvature or roughness of the surface has influences on cell migration (He and Jiang, 2017; Pieuchot et al., 2018).

In our experiments, we designed a novel setting of transwell holders and cover glasses to create a regular and consistent wound area on soft hydrogel surface without altering the curvature or roughness of the hydrogel surface (Fig. 3.8a). And the reason for choosing 3D-printed Polylactic Acid (PLA) transwell holder is that PLA holder is flexible enough to insert cover glasses. Also,

the cell density was maintained at a high cell setting density ($5 \times 10^5 \text{ cm}^{-2}$) on the surface of alginate hydrogels to get monolayer of NIH/3T3-GFP cells. Also, due to the limited time, we only tried three conditions of gels (Table 3.4): I1G-Original, 7 kPa (slow stress relaxation with high elastic modulus); I1G-3Mrad, 7 kPa (fast stress relaxation with high elastic modulus); I1G-Original, 17 kPa (slow stress relaxation with high elastic modulus). According to the results, 2D collective cell migration was suppressed by both high elastic modulus and slow relaxation rate, which may also because of the influence of stiffness and stress relaxation on the formation of focal adhesions. The size of focal adhesions increases to the range that suppress the 2D collective cell migration, under the effects of high elastic modulus and slow relaxation rate.

4.4 3D Single Cell Tracking

There have been extensive studies on the mechanisms and regulation of cell migration in 2D models. However, the natural substrate for most of cells in living tissue is 3D structure, and is complex and dynamic in the molecular composition as well, which has led up to increasing number of studies to switch to 3D models (Decaestecker et al., 2006). 3D models have some advantages over 2D models in simulating *in vivo* situations and 3D models enable researchers to study specific factors such as cell polarity, dimensionality and architecture under more biologic conditions (Even-Ram and Yamada, 2005). In 3D substrates, cell migration includes both cell movement and the remodeling of the matrix (Decaestecker et al., 2006). There are several parameters play critical roles in 3D migration: mechanical properties, the pore size and the cell

density (Wu, Gilkes, and Wirtz, 2018).

The study of 3D migration is more complex than studies in 2D migration and usually consists of the utilization of 3D matrices as a scaffold. Also compared with 2D cell tracking, along with (t, X, Y) locations, 3D cell tracking needs to record the cell movement on Z-axis as well. However, most previous 3D migration studies only observed cell movements in a fixed focal plane and cell movements in Z direction are usually ignored (Wu, Gilkes, and Wirtz, 2018). This negligence is rational since the assumptions for 3D migration are that cell movements are isotropic in x, y , and z axes and migrations of each direction are equivalent to each other.

Therefore, in our experiments, we combined the time-lapse microscopy with Z-stack function to capture the cell migration, which allows us to gain more data on several fixed focal plane. What's more, according to previous studies, cell density (the number of cell per unit volume in matrix) plays a critical role in 3D cell migration and cell speed increases $> 50\%$ when cell density passes a threshold of $5 \times 10^4 \text{ ml}^{-1}$ (Wu, Gilkes, and Wirtz, 2018). Therefore, we kept the cell density constant at 10^6 ml^{-1} to exclude the influence of the cell density variation and the high cell density we used would enhance the 3D cell migration due to paracrine signaling but kept the cells in single models, which made it easier to capture and measure the cell movements. In our studies, NIH/3T3-GFP cells were encapsulated within RGD-coupled alginate gels with slow stress relaxation rate (I1G-Original) and fast stress relaxation rate (I1G-8 Mrad) while keeping the elastic modulus at 7 kPa. Based on the results, the average speed of 3D single cell migration was suppressed within

the gels with long timescale for stress relaxation ($\tau_{1/2} > 1h$) (Fig. 3.9 and Table 3.5).

There were previous studies representing the relationship between matrix stiffness and 3D cell migration, and the genes expression related to cell-matrix adhesion was influenced by stiffness (Ehrbar et al., 2011; Lange and Fabry, 2013; Schwartz and DeSimone, 2008; Darnell, Gu, and Mooney, 2018). So the genes expression related to cell-matrix adhesion or cell migration could also be influenced by stress relaxation rates and the fast relaxation rate may promote the genes expression correlated with 3D single cell migration.

4.5 3D Radial Cell Migration

3D radial cell migration assay is one of the widely used models to study 3D collective cell migration properties and is one of the perfectly suitable models to study tumor invasion (Decaestecker et al., 2006). In general, there are two steps of 3D Radial cell migration. First, generate concentrations or clusters of cells, then embed cells in a thin-layer of substrates (Decaestecker et al., 2006; Cisneros Castillo et al., 2016). As compared to the other two dimensions, the thickness of the hydrogels is low, the quantification of cell migration is usually based on the projection of cell locations on the horizontal plane (Decaestecker et al., 2006). Also, there are several advantages of 3D radial cell migration assay compared with other 3D collective models (Decaestecker et al., 2006; Vernon and Sage, 1999). First, compared to transwell cell culture chamber assay, cells do not need to migrate through a porous membrane. Then the optical properties of alginate hydrogels, i.e. the transparent nature, allows the

observations of cells migration. Also, compared to the gel invasion assay, it is relatively simple for the cell migration to be quantified (e.g. the number of migrated cells, the distances from original sites and the area of spread) using radial assay.

In our experiments, we studied nine different mechanical properties gel conditions in order to fully understand the influences of both stiffness and stress relaxation to 3D radial cell migration (Fig.3.14, 3.15 and 3.16). Also, we used spheroids with the same size (5000 cells/spheroid, $\sim 300\ \mu\text{m}$) in all the experiments to exclude the influence of spheroids' initial size (Cisneros Castillo et al., 2016). And the results showed the 3D radial cell migration mode of NIH/3T3-GFP cells was protrusion pattern in alginate hydrogels and the protrusion growth was suppressed within the gels with slow stress relaxation rate and high stiffness. Based on previous studies, substrate properties including stiffness, substrate stress relaxation rate have been shown to influences on gene expression (Darnell, Gu, and Mooney, 2018; Darnell et al., 2018). When cells were cultured on substrates with various micro-environmental features, cells would need to integrate different biophysical inputs (Darnell et al., 2018). And different material features or environments can induce parallel effects (Darnell et al., 2018). Therefore, it might be the reasons why the growth of protrusions could be suppressed by both slow stress relaxation and high stiffness.

However, the stress relaxation might be a more critical factor for the 3D radial protrusion migration than the elastic modulus under the tested conditions. Because there was no obvious migration when the stress relaxation half

time ($\tau_{1/2}$) of alginate hydrogels was longer than 4 min. Therefore, there might be a critical value or threshold of stress relaxation half time ($\tau_{1/2}$) to allow the growth of protrusions and the critical value might be between 240s and 170s (Table 3.2). However, when relaxation half time ($\tau_{1/2}$) of alginate hydrogels was less than 3 min, there were protrusion growth for all three different stiffness conditions (Fig. 3.15). But higher the elastic modulus of alginate hydrogels, shorter the average length of the longest protrusions (Fig.3.16). The results of 3D collective cell migration were basically in accordance with the results of 3D single cell migration.

References

- Fallis, a.G (2013). *Cell migration*. Vol. 53. 9, pp. 1689–1699. ISBN: 9788578110796. DOI: [10.1017/CB09781107415324.004](https://doi.org/10.1017/CB09781107415324.004). arXiv: [arXiv:1011.1669v3](https://arxiv.org/abs/1011.1669v3).
- Lauffenburger, Douglas A. and Alan F. Horwitz (1996). “Cell migration: A physically integrated molecular process”. In: *Cell* 84.3, pp. 359–369. ISSN: 00928674. DOI: [10.1016/S0092-8674\(00\)81280-5](https://doi.org/10.1016/S0092-8674(00)81280-5).
- Decaestecker, Christine, Olivier Debeir, Philippe Van Ham, and Robert Kiss (2006). “Can Anti-Migratory Drugs Be Screened InVitro ? A Review of 2D and 3D Assays for the Quantitative Analysis of Cell Migration”. In: 27.2, pp. 149–176. DOI: [10.1002/med.20078](https://doi.org/10.1002/med.20078).
- Lintz, Marianne, Adam Muñoz, and Cynthia A. Reinhart-King (2017). “The Mechanics of Single Cell and Collective Migration of Tumor Cells”. In: *Journal of Biomechanical Engineering* 139.2, p. 021005. ISSN: 0148-0731. DOI: [10.1115/1.4035121](https://doi.org/10.1115/1.4035121). URL: <http://www.ncbi.nlm.nih.gov/pubmed/27814431><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5395914><http://biomechanical.asmedigitalcollection.asme.org/article.aspx?doi=10.1115/1.4035121>.
- Friedl, Peter and Darren Gilmour (2009). “Collective cell migration in morphogenesis, regeneration and cancer”. In: *Nature Reviews Molecular Cell Biology* 10.7, pp. 445–457. ISSN: 1471-0072. DOI: [10.1038/nrm2720](https://doi.org/10.1038/nrm2720). URL: <http://www.nature.com/articles/nrm2720>.
- Duda, Dan G, Annique M M J Duyverman, Mitsutomo Kohno, Matija Snuderl, Ernst J A Steller, Dai Fukumura, and Rakesh K Jain (2010). “Malignant cells facilitate lung metastasis by bringing their own soil.” In: *Proceedings of the National Academy of Sciences of the United States of America* 107.50, pp. 21677–82. ISSN: 1091-6490. DOI: [10.1073/pnas.1016234107](https://doi.org/10.1073/pnas.1016234107). URL: <http://www.ncbi.nlm.nih.gov/pubmed/21098274><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3003109>.
- Horssen, Remco van and Timo L.M. ten Hagen (2011). “Crossing barriers: The new dimension of 2D cell migration assays”. In: *Journal of Cellular*

- Physiology* 226.1, pp. 288–290. ISSN: 00219541. DOI: 10.1002/jcp.22330. URL: <http://doi.wiley.com/10.1002/jcp.22330>.
- Vernon, Robert B and Michel D Gooden (2002). “New technologies in vitro for analysis of cell movement on or within collagen gels.” In: *Matrix biology : journal of the International Society for Matrix Biology* 21.8, pp. 661–9. ISSN: 0945-053X. DOI: 10.1016/S0945-053X(02)00091-4. URL: <http://www.ncbi.nlm.nih.gov/pubmed/12524052>.
- Pelham, Robert J, J R And, and Yu-Li Wang (1998). *Cell Locomotion and Focal Adhesions Are Regulated by the Mechanical Properties of the Substrate*. Tech. rep., pp. 348–350. URL: <http://www.journals.uchicago.edu/t-and-c>.
- Engler, Adam, Lucie Bacakova, Cynthia Newman, Alina Hategan, Maureen Griffin, and Dennis Discher (2004). “Substrate Compliance versus Ligand Density in Cell on Gel Responses”. In: *Biophysical Journal* 86.1, pp. 617–628. ISSN: 0006-3495. DOI: 10.1016/S0006-3495(04)74140-5. URL: <https://www.sciencedirect.com/science/article/pii/S0006349504741405>.
- Lo, C M, H B Wang, M Dembo, and Y L Wang (2000). “Cell movement is guided by the rigidity of the substrate.” In: *Biophysical journal* 79.1, pp. 144–52. ISSN: 0006-3495. DOI: 10.1016/S0006-3495(00)76279-5. URL: <http://www.ncbi.nlm.nih.gov/pubmed/10866943><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1300921>.
- Wang, Peng-Yuan, Wei-Bor Tsai, and Nicolas H. Voelcker (2012). “Screening of rat mesenchymal stem cell behaviour on polydimethylsiloxane stiffness gradients”. In: *Acta Biomaterialia* 8.2, pp. 519–530. ISSN: 1742-7061. DOI: 10.1016/J.ACTBIO.2011.09.030. URL: <https://www.sciencedirect.com/science/article/pii/S1742706111004235>.
- Deroanne, C, Charles M. Lapiere, and Betty V. Nusgens (2001). “In vitro tubulogenesis of endothelial cells by relaxation of the coupling extracellular matrix-cytoskeleton”. In: *Cardiovascular Research* 49.3, pp. 647–658. ISSN: 00086363. DOI: 10.1016/S0008-6363(00)00233-9. URL: [https://academic.oup.com/cardiovasres/article-lookup/doi/10.1016/S0008-6363\(00\)00233-9](https://academic.oup.com/cardiovasres/article-lookup/doi/10.1016/S0008-6363(00)00233-9).
- Flanagan, Lisa A., Liza M. Rebaza, Stanislava Derzic, Philip H. Schwartz, and Edwin S. Monuki (2006). “Regulation of human neural precursor cells by laminin and integrins”. In: *Journal of Neuroscience Research* 83.5, pp. 845–856. ISSN: 0360-4012. DOI: 10.1002/jnr.20778. URL: <http://www.ncbi.nlm.nih.gov/pubmed/16477652><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2409144><http://doi.wiley.com/10.1002/jnr.20778>.

- Engler, Adam J., Shamik Sen, H. Lee Sweeney, and Dennis E. Discher (2006a). "Matrix Elasticity Directs Stem Cell Lineage Specification". In: *Cell* 126.4, pp. 677–689. ISSN: 00928674. DOI: [10.1016/j.cell.2006.06.044](https://doi.org/10.1016/j.cell.2006.06.044). URL: <http://www.ncbi.nlm.nih.gov/pubmed/16923388><https://linkinghub.elsevier.com/retrieve/pii/S0092867406009615>.
- Huebsch, Nathaniel, Praveen R Arany, Angelo S Mao, Dmitry Shvartsman, Omar A Ali, Sidi A Bencherif, José Rivera-Feliciano, and David J Mooney (2010). "Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate." In: *Nature materials* 9.6, pp. 518–26. ISSN: 1476-1122. DOI: [10.1038/nmat2732](https://doi.org/10.1038/nmat2732). URL: <http://www.nature.com/articles/nmat2732><http://www.ncbi.nlm.nih.gov/pubmed/20418863><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2919753>.
- Discher, Dennis E., David J. Mooney, and Peter W. Zandstra (2009). "Growth Factors, Matrices, and Forces Combine and Control Stem Cells". In: *Science* 324.5935, pp. 1673–1677. ISSN: 0036-8075. DOI: [10.1126/SCIENCE.1171643](https://doi.org/10.1126/SCIENCE.1171643). URL: <https://science.sciencemag.org/content/324/5935/1673>.
- Chaudhuri, Ovijit, Luo Gu, Darinka Klumpers, Max Darnell, Sidi A. Bencherif, James C. Weaver, Nathaniel Huebsch, Hong-pyo Lee, Evi Lippens, Georg N. Duda, and David J. Mooney (2016). "Hydrogels with tunable stress relaxation regulate stem cell fate and activity". In: *Nature Materials* 15.3, pp. 326–334. ISSN: 1476-1122. DOI: [10.1038/nmat4489](https://doi.org/10.1038/nmat4489). URL: <http://www.nature.com/articles/nmat4489>.
- Chaudhuri, Ovijit, Luo Gu, Max Darnell, Darinka Klumpers, Sidi A. Bencherif, James C. Weaver, Nathaniel Huebsch, and David J. Mooney (2015). "Substrate stress relaxation regulates cell spreading". In: *Nature Communications* 6.1, p. 6365. ISSN: 2041-1723. DOI: [10.1038/ncomms7365](https://doi.org/10.1038/ncomms7365). URL: <http://www.nature.com/articles/ncomms7365>.
- Cameron, Andrew. R., Jessica. E. Frith, and Justin. J. Cooper-White (2011). "The influence of substrate creep on mesenchymal stem cell behaviour and phenotype". In: *Biomaterials* 32.26, pp. 5979–5993. ISSN: 0142-9612. DOI: [10.1016/J.BIOMATERIALS.2011.04.003](https://doi.org/10.1016/J.BIOMATERIALS.2011.04.003). URL: <https://www.sciencedirect.com/science/article/pii/S0142961211003851>.
- Cameron, Andrew R., Jessica E. Frith, Guillermo A. Gomez, Alpha S. Yap, and Justin J. Cooper-White (2014). "The effect of time-dependent deformation of viscoelastic hydrogels on myogenic induction and Rac1 activity in mesenchymal stem cells". In: *Biomaterials* 35.6, pp. 1857–1868. ISSN:

- 0142-9612. DOI: [10.1016/J.BIOMATERIALS.2013.11.023](https://doi.org/10.1016/J.BIOMATERIALS.2013.11.023). URL: <https://www.sciencedirect.com/science/article/pii/S0142961213013768>.
- Graessley, William W. (1982). "Entangled linear, branched and network polymer systems – Molecular theories". In: *Synthesis and Degradation Rheology and Extrusion*. Berlin/Heidelberg: Springer-Verlag, pp. 67–117. DOI: [10.1007/BFb0038532](https://doi.org/10.1007/BFb0038532). URL: <http://www.springerlink.com/index/10.1007/BFb0038532>.
- Hadden, William J., Jennifer L. Young, Andrew W. Holle, Meg L. McFetridge, Du Yong Kim, Philip Wijesinghe, Hermes Taylor-Weiner, Jessica H. Wen, Andrew R. Lee, Karen Bieback, Ba-Ngu Vo, David D. Sampson, Brendan F. Kennedy, Joachim P. Spatz, Adam J. Engler, and Yu Suk Choi (2017). "Stem cell migration and mechanotransduction on linear stiffness gradient hydrogels". In: *Proceedings of the National Academy of Sciences* 114.22. ISSN: 0027-8424. DOI: [10.1073/pnas.1618239114](https://doi.org/10.1073/pnas.1618239114).
- Rowley, Jon A., Gerard Madlambayan, and David J. Mooney (1999). "Alginate hydrogels as synthetic extracellular matrix materials". In: *Biomaterials* 20.1, pp. 45–53. ISSN: 0142-9612. DOI: [10.1016/S0142-9612\(98\)00107-0](https://doi.org/10.1016/S0142-9612(98)00107-0). URL: <https://www.sciencedirect.com/science/article/pii/S0142961298001070>.
- Ascione, Flora, Angela Vasaturo, Sergio Caserta, Vittoria D'Esposito, Pietro Formisano, and Stefano Guido (2016). "Comparison between fibroblast wound healing and cell random migration assays in vitro". In: *Experimental Cell Research* 347.1, pp. 123–132. ISSN: 10902422. DOI: [10.1016/j.yexcr.2016.07.015](https://doi.org/10.1016/j.yexcr.2016.07.015). URL: <http://dx.doi.org/10.1016/j.yexcr.2016.07.015>.
- Cukierman, E, R Pankov, D R Stevens, and K M Yamada (2001). "Taking cell-matrix adhesions to the third dimension." In: *Science (New York, N.Y.)* 294.5547, pp. 1708–12. ISSN: 0036-8075. DOI: [10.1126/science.1064829](https://doi.org/10.1126/science.1064829). URL: <http://www.sciencemag.org/cgi/doi/10.1126/science.1064829><http://www.ncbi.nlm.nih.gov/pubmed/11721053>.
- Bershadsky, Alexander D., Nathalie Q. Balaban, and Benjamin Geiger (2003). "Adhesion-Dependent Cell Mechanosensitivity". In: *Annual Review of Cell and Developmental Biology* 19.1, pp. 677–695. ISSN: 1081-0706. DOI: [10.1146/annurev.cellbio.19.111301.153011](https://doi.org/10.1146/annurev.cellbio.19.111301.153011). URL: <http://www.ncbi.nlm.nih.gov/pubmed/14570586><http://www.annualreviews.org/doi/10.1146/annurev.cellbio.19.111301.153011>.
- Engler, Adam J., Shamik Sen, H. Lee Sweeney, and Dennis E. Discher (2006b). "Matrix Elasticity Directs Stem Cell Lineage Specification". In: *Cell* 126.4,

- pp. 677–689. ISSN: 0092-8674. DOI: 10.1016/J.CELL.2006.06.044. URL: <https://www.sciencedirect.com/science/article/pii/S0092867406009615>.
- Sugawara, Michiko, Hiromi Miyoshi, Takuya Miura, Hiroto Tanaka, Ken Ichi Tsubota, and Hao Liu (2016). “Dynamics of Actin Stress Fibers and Focal Adhesions during Slow Migration in Swiss 3T3 Fibroblasts: Intracellular Mechanism of Cell Turning”. In: *BioMed Research International*. ISSN: 23146141. DOI: 10.1155/2016/5749749.
- De Pascalis, Chiara and Sandrine Etienne-Manneville (2017). “Single and collective cell migration: the mechanics of adhesions”. In: 28. DOI: 10.1091/mbc.E17-03-0134. URL: <https://www.molbiolcell.org/doi/pdf/10.1091/mbc.e17-03-0134>.
- Kim, Dong-Hwee and Denis Wirtz (2013b). “Predicting how cells spread and migrate: focal adhesion size does matter.” In: *Cell adhesion & migration* 7.3, pp. 293–6. ISSN: 1933-6926. DOI: 10.4161/cam.24804. URL: <http://www.tandfonline.com/doi/abs/10.4161/cam.24804><http://www.ncbi.nlm.nih.gov/pubmed/23628962><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3711996>.
- Kim, Dong-Hwee and Denis Wirtz (2013a). “Focal adhesion size uniquely predicts cell migration”. In: *The FASEB Journal* 27.4, pp. 1351–1361. ISSN: 0892-6638. DOI: 10.1096/fj.12-220160. URL: <http://www.fasebj.org/doi/10.1096/fj.12-220160>.
- Wu, Pei-Hsun, Daniele M. Gilkes, and Denis Wirtz (2018). “The Biophysics of 3D Cell Migration”. In: *Annual Review of Biophysics* 47.1, pp. 549–567. ISSN: 1936-122X. DOI: 10.1146/annurev-biophys-070816-033854. URL: <https://www.annualreviews.org/doi/10.1146/annurev-biophys-070816-033854>.
- Falanga, Vincent (2005). “Wound healing and its impairment in the diabetic foot”. In: *The Lancet* 366.9498, pp. 1736–1743. ISSN: 0140-6736. DOI: 10.1016/S0140-6736(05)67700-8. URL: <https://www.sciencedirect.com/science/article/pii/S0140673605677008>.
- P., Bainbridge (2013). “Wound healing and the role of fibroblasts”. In: *Journal of Wound Care* 22.8, pp. 407–412. ISSN: 0969-0700. DOI: 10.12968/jowc.2013.22.8.407. URL: <http://www.ncbi.nlm.nih.gov/pubmed/23924840><http://www.magonlinelibrary.com/doi/10.12968/jowc.2013.22.8.407>.
- Rodriguez, Luis G., Xiaoyang Wu, and Jun-Lin Guan. “Wound-Healing Assay”. In: *Cell Migration* 294, pp. 023–030. ISSN: 1064-3745. DOI: 10.1385/1-59259-860-9:023. URL: <http://link.springer.com/10.1385/1-59259-860-9:023>.

- Grada, Ayman, Marta Otero-Vinas, Francisco Prieto-Castrillo, Zaidal Obagi, and Vincent Falanga (2017). "Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay". In: *Journal of Investigative Dermatology* 137.2, e11–e16. ISSN: 15231747. DOI: [10.1016/j.jid.2016.11.020](https://doi.org/10.1016/j.jid.2016.11.020). URL: <http://dx.doi.org/10.1016/j.jid.2016.11.020>.
- He, Xiuxiu and Yi Jiang (2017). "Substrate curvature regulates cell migration". In: *Physical Biology* 14.3, p. 035006. ISSN: 1478-3975. DOI: [10.1088/1478-3975/aa6f8e](https://doi.org/10.1088/1478-3975/aa6f8e). URL: <http://www.ncbi.nlm.nih.gov/pubmed/28535145><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5572487><http://stacks.iop.org/1478-3975/14/i=3/a=035006?key=crossref.88ed625e87aadead54959949fc9abc8b>.
- Pieuchot, Laurent, Julie Marteau, Alain Guignandon, Thomas Dos Santos, Isabelle Brigaud, Pierre-François Chauvy, Thomas Cloatre, Arnaud Ponche, Tatiana Petithory, Pablo Rougerie, Maxime Vassaux, Jean-Louis Milan, Nayana Tusamda Wakhloo, Arnaud Spangenberg, Maxence Bigerelle, and Karine Anselme (2018). "Curvotaxis directs cell migration through cell-scale curvature landscapes". In: *Nature Communications* 9.1, p. 3995. ISSN: 2041-1723. DOI: [10.1038/s41467-018-06494-6](https://doi.org/10.1038/s41467-018-06494-6). URL: <http://www.ncbi.nlm.nih.gov/pubmed/30266986><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6162274><http://www.nature.com/articles/s41467-018-06494-6>.
- Even-Ram, Sharona and Kenneth M. Yamada (2005). "Cell migration in 3D matrix". In: *Current Opinion in Cell Biology* 17.5, pp. 524–532. ISSN: 09550674. DOI: [10.1016/j.ceb.2005.08.015](https://doi.org/10.1016/j.ceb.2005.08.015). URL: <http://linkinghub.elsevier.com/retrieve/pii/S0955067405001183>.
- Ehrbar, M., A. Sala, P. Lienemann, A. Ranga, K. Mosiewicz, A. Bittermann, S.C. Rizzi, F.E. Weber, and M.P. Lutolf (2011). "Elucidating the Role of Matrix Stiffness in 3D Cell Migration and Remodeling". In: *Biophysical Journal* 100.2, pp. 284–293. ISSN: 0006-3495. DOI: [10.1016/J.BPJ.2010.11.082](https://doi.org/10.1016/J.BPJ.2010.11.082). URL: <https://www.sciencedirect.com/science/article/pii/S0006349510051891>.
- Lange, Janina R. and Ben Fabry (2013). *Cell and tissue mechanics in cell migration*. DOI: [10.1016/j.yexcr.2013.04.023](https://doi.org/10.1016/j.yexcr.2013.04.023). arXiv: [NIHMS150003](https://arxiv.org/abs/NIHMS150003).
- Schwartz, Martin A and Douglas W DeSimone (2008). "Cell adhesion receptors in mechanotransduction". In: *Current Opinion in Cell Biology* 20.5, pp. 551–556. ISSN: 09550674. DOI: [10.1016/j.ceb.2008.05.005](https://doi.org/10.1016/j.ceb.2008.05.005). URL: <http://www.ncbi.nlm.nih.gov/pubmed/18583124><http://www.pubmedcentral>.

- [nih.gov/articlerender.fcgi?artid=PMC2581799https://linkinghub.elsevier.com/retrieve/pii/S0955067408000860](https://linkinghub.elsevier.com/retrieve/pii/S0955067408000860).
- Darnell, Max, Luo Gu, and David Mooney (2018). "RNA-seq reveals diverse effects of substrate stiffness on mesenchymal stem cells." In: *Biomaterials* 181, pp. 182–188. ISSN: 1878-5905. DOI: 10.1016/j.biomaterials.2018.07.039. URL: <https://linkinghub.elsevier.com/retrieve/pii/S0142961218305246><http://www.ncbi.nlm.nih.gov/pubmed/30086447><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6258186>.
- Cisneros Castillo, Liliana R., Andrei Dumitru Oancea, Christian Stüllein, and Anne Régnier-Vigouroux (2016). "Evaluation of Consistency in Spheroid Invasion Assays". In: *Scientific reports* 6, June, p. 28375. ISSN: 20452322. DOI: 10.1038/srep28375. URL: <http://dx.doi.org/10.1038/srep28375>.
- Vernon, Robert B. and E. Helene Sage (1999). "A Novel, Quantitative Model for Study of Endothelial Cell Migration and Sprout Formation within Three-Dimensional Collagen Matrices". In: *Microvascular Research* 57.2, pp. 118–133. ISSN: 0026-2862. DOI: 10.1006/MVRE.1998.2122. URL: <https://www.sciencedirect.com/science/article/abs/pii/S0026286298921227>.
- Darnell, Max, Alison O'Neil, Angelo Mao, Luo Gu, Lee L Rubin, and David J Mooney (2018). "Material microenvironmental properties couple to induce distinct transcriptional programs in mammalian stem cells." In: *Proceedings of the National Academy of Sciences of the United States of America* 115.36, E8368–E8377. ISSN: 1091-6490. DOI: 10.1073/pnas.1802568115. URL: <http://www.ncbi.nlm.nih.gov/pubmed/30120125><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6130338>.

Chapter 5

Conclusion and Future Work

In conclusion, these findings indicate that parallel to stiffness, substrate stress relaxation might also be a critical mechanical properties of matrix that has impacts on cell migration, including 2D single cell migration, 2D collective cell migration, 3D single cell migration and 3D collective cell migration. 2D collective cell migration, 3D single cell migration and 3D collective cell migration are all enhanced by fast relaxation substrates, while keeping stiffness constant. However, when increasing the stress relaxation rate, 2D single cell migration speed first increases, then decreases.

For future works, we still need to collect more data to confirm the results we got. Also, we need to design experiments to study the mechanisms of the impact of stress relaxation on cell migration. It is also interesting to try different cell lines for the migration experiments, such as cancer cells or stem cells. Specifically, for the 3D single cell migration assay, the small pore size of alginate hydrogels ($\sim 5\text{nm}$) (Lee and Mooney, 2012) makes it harder for cells to migrate within the hydrogels which increases the difficulty to analyze the movements of cells. Therefore, we can decrease the polymer concentration

of the alginate hydrogels to increase the pore size, in order to enhance the phenomena of 3D single cell migration.

References

Lee, Kuen Yong and David J. Mooney (2012). "Alginate: Properties and biomedical applications". In: *Progress in Polymer Science* 37.1, pp. 106–126. ISSN: 0079-6700. DOI: [10.1016/J.PROGPOLYMSCI.2011.06.003](https://doi.org/10.1016/J.PROGPOLYMSCI.2011.06.003). URL: <https://www.sciencedirect.com/science/article/pii/S0079670011000918>.

Yi Zuo (Jan. 29th1996, China)
(1)410-570-9573/ yzuo6@jhu.edu

EDUCATION

Johns Hopkins University

M. S. E. in Materials Science & Engineering

- GPA: 3.74/4.0
- Thesis: Hydrogels with Tunable Stress Relaxation Regulate Stem Cell Migration

Sep.2017-Present

Baltimore, USA

Central South University

B.E. in Materials Chemistry

- GPA: 89.22/100 3.81/4.0 Ranking:2/95

Sep.2013-Jun. 2017

Hunan, China

RESEARCH EXPERIENCES

Hydrogels with Tunable Stress Relaxation Regulate Stem Cell Migration

Master's Program

Adviser: Prof. Luo Gu

- Develop a materials approach to tune the rate of stress relaxation of hydrogels, independently of the hydrogel's initial elastic modulus, degradation, and cell-adhesion-ligand density
- Build 2D & 3D models for single cell migration & population migration
- Analyze the relationship between substrate stress relaxation and cell migration

Sep.2017-Present

Baltimore, USA

Summer RA in Johns Hopkins Institute for NanoBioTechnology (INBT)

- Optimized 2D & 3D cell culture and cell migration models
- Mice & Rats dissection and organ harvesting
- Maintained and operated GPC (Gel permeation chromatography)
- Commitment: 40 hours/week

Jun.2018—Aug.2018

Baltimore, USA

Fabrication and Formaldehyde Gas Sensing Property of Porous Cd-Doped In₂O₃ Nanospheres

Independent Research Program

Adviser: Prof. Xueping Gan

- Prepared spherical In₂O₃ particles with uniform dispersity and porous structure by a hydrothermal and calcination process.
- Doped In₂O₃ nanospheres with Cd to improve the sensitivity and decrease optimum temperature as gas sensors.

Jun. 2015-Jun.2016

Hunan, China

WORK EXPERIENCES

2019 Spring RA for Biomaterials Lab

Jan.2019-Present

Baltimore, USA

Undergraduate Design Project Instructor

Sep.2018-Present

Baltimore, USA

2018 Fall TA for *Biomaterials II: Host response and biomaterials applications*

Sep.2018-Dec.2018

Baltimore, USA

Danyang Guest, Keen & Neetleolds Co., Ltd.

Summer Intern

- Dealt with data, materials and acquired product quality testing methods in Dept. of Quality
- In charge of foreign guests' reception, drafted event manuscripts and refreshed staff cards in Dept. of Personnel

Jun. 2016- July 2016

Danyang, China

PUBLICATION

Wang, J; Zuo, Y; Gao, C; Gan, X; Zhou, K; Li, Z. (2017). "Fabrication and formaldehyde gas sensing property of porous Cd-doped In₂O₃ nanospheres." *Materials Science and Engineering of Powder Metallurgy*. 22. 372-377.

MISCELLANEOUS

Languages: English (Fluent), Mandarin Chinese (Native), Japanese (Conversational)

Software: Word, PowerPoint, Excel, Mathematica, Origin, MATLAB

Skills: ▪ Materials Characterization: GPC (Gel permeation chromatography), NMR (Nuclear magnetic resonance), SEM (Scanning Electron Microscope), Confocal Microscope, Fluorescence Microscope, Tensile Tester, Nano-indenter, Rheometer;

- Cell Culture: 2D cell culture & 3D cell culture
- Rodent Dissection & Injection